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(54) Title: AGENTS AND METHODS FOR MODULATION OF ZINC TRANSFER BY METALLOTHIONEIN

(57) Abstract

A method is provided for the treatment of diseases of plants and animals where such diseases involve disturbance of zinc homeostasis. The method involves administration of therapeutic agents that after cellular oxidation potential so as to oxidize metallothionein and thereby release zinc ions, or as to maintain metallothionein in a reduced state so as to prevent transfer of zinc from metallothionein to zinc acceptors.

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AGENTS AND METHODS FOR MODULATION OF ZINC TRANSFER BY METALLOTHIONEIN

This application claims priority to U.S. provisional applications Serial Number 60/079,969, filed March 30, 1998, and Serial Number 60/084,953, filed May 11, 1998.

1. INTRODUCTION

The present invention relates to control of zinc metabolism mediated by metallothioneins. Metallothioneins are both the predominant cellular reservoir for bioavailable zinc and the primary shuttle for zinc transfer among biomolecules. Agents are provided that enhance or prevent the release of zinc from metallothioneins and thereby affect metabolic pathways that depend on the availability or uptake of zinc.

2. BACKGROUND OF THE INVENTION

2.1. METALLOTHIONEIN

Metallothionein (MT) was discovered in 1957 (Margoshes, M. and Vallee, B. L., 1957, J. Am. Chem. Soc. 79, 4813). By all counts it is a most unusual and unconventional protein (Vallee, B.L., 1979, Experientia Suppl. 34, 19-40; Vallee, B.L., 1987, Experientia Suppl. 52, 5-16; Vallee, B. L., 1991, Meth. Enzymol. 205, 3-7; Vallee, B.L. and Maret, W., 1993, in Metallothionein III, eds. Suzuki, K. T., Imura, N. & Kimura, M. (Birkhäuser, Basel), 1; Vallee, 1995, Neurochem. Interntl. 27, 23). One third of its 60+ amino acids are cysteines and eight are lysines. It contains neither aromatic amino acids nor histidine. MT usually binds seven zinc atoms, but it can also contain copper, cadmium, iron and traces of other metals. In an evolutionary sense it is a very old protein. The composition of its two major isoproteins has changed only imperceptibly over time. The number of genes that code for human MTs could be as high as 17. MT-1 and MT-2 are the two prevalent forms, which are expressed, but whose physiological functions are unknown. MT-3 was discovered only recently in brains from patients afflicted with Alzheimer's disease (Uchida, Y., Takio, K., Titani, K., Ihara, Y., and Tomonaga, M. (1991) Neuron 7, 337). Its discovery was based on the fact that it inhibits the growth of neurons. Thus far, it is the only MT that is known to exhibit such a specific biological function. This isoform contains zinc and copper(I), but not cadmium or other metals. Multiple factors among them members of the nuclear hormone receptor family, interferons, inducers of the acute phase response, and metalloregulatory proteins, affect tissue- and isoprotein-specific gene

expression. In addition there are numerous other agents that induce it but whose signalling pathways remain obscure. Thionein, the apoform of MT has never been isolated as such from any biological material. Apparently, upon its formation, it instantaneously combines with zinc, whose "free" concentration in the cell has been reported to be exceedingly low, i.e. in the nanomolar to picomolar range. All these facts suggest that MT must be biologically essential. This, indeed, has now been proven to be correct. MT-1/-2 knock-out mice become obese, demonstrating the involvement of MT in energy metabolism (Beattie, J. H, Wood, A. M., Newman, A. M., Bremner, I., Choo, K. H. A., Michalska, A. E., Duncan, J. S., and Trayhurn, P., 1998, Proc. Natl. Acad. Sci. USA 95, 358).

While MT was discovered 40 years ago, the 3D structure of MT by both X-ray crystallography (Robbins, A. H. and Stout, C. D., 1991, Meth. Enzymol. 205, 485) and NMR spectroscopy (Wüthrich, K., 1991, Meth. Enzymol. 205, 502) has been reported only seven years ago. The structure has proved critical to efforts designed to establish its function. The protein has the shape of a dumb-bell and envelops the metals which it contains in two separate domains in a manner which effectively shields them from the environment. It is most remarkable that the metals are arranged in a cluster structure unique to biology. In one cluster four atoms are bound to eleven cysteines, five of which bridge the metals, and the other has three metal atoms and nine cysteines with three bridges.

The biological importance of zinc does not require elaboration at this point, since catalytic functions of zinc in enzymes and its structural functions in zinc finger proteins have been documented amply (Vallee, B. L. and Falchuk, K. F., 1993, Physiol. Rev. 73, 79-118; Vallee, B. L. and Auld, D. S., 1993, Acc. Chem. Res. 26, 543). Zinc in MT is bound extremely tightly (K_D about 10^{-13} M) (Kägi, J. H. R., 1993, in *Metallothionein III*, eds. Suzuki, K. T., Imura, N. & Kimura, M. (Birkhäuser, Basel), pp. 29). There have not been any indications how it is removed or added.

Findings that cast light on the circumstances under which zinc is released from MT have been reported. For example, the oxidative metal release from MT by glutathione disulfide has been reported (Maret, W., 1994, Proc. Natl. Acad. Sci. USA 91, 237), and exchange between MT and the zinc cluster in the Gal4 transcription factor has been demonstrated (Maret, W., Larsen, K. S., and Vallee, B. L., 1997, Proc. Natl. Acad. Sci. USA 94, 2233). These previous reports, however, do not report or suggest the methods and compositions of the present invention, and in fact, failed to appreciate the synergistic release of zinc by oxidants in the presence of glutathione, and failed to provide for zinc transfer on a physiologically significant time scale.

Zinc, in contrast to copper or iron, is redox-inert. Hence, the properties of zinc complexes are not altered by valence changes of the central atom in a manner akin to those in iron or copper complexes. However, the redox state of the sulfur ligands can be changed.

2.2. DISORDERS OF THE CENTRAL NERVOUS SYSTEM

2.2.1. ALZHEIMER'S DISEASE

The cerebral cortexes of Alzheimer's disease ("AD") patients contain neurofibrillary tangles and senile (amyloid) plaques (D. M. A. Mann *et al.*, 1988, Neuropathol. Appl. Neurol., 14, 177). These structures are associated with the dementia and memory loss that are characteristic of the disease.

2.2.2. PARKINSON'S DISEASE

Parkinson's disease involves degeneration of pigmented neuronal systems in the brain stem that leads to neuromediator dysfunction. The principal cytoskeletal pathology associated with Parkinson's disease is the Lewy body which predominately occurs in aminergic and other subcortical, spinal cord, and sympathetic ganglia neurons, and also to a lesser extent in the cerebral cortex. Lewy bodies in Parkinson's disease lead to a degeneration of the dopaminergic pathway of the pigmented neuronal systems as well as to a degeneration of other neuronal systems, and this degeneration leads to a complex set of functional deficits (M. Ebadi *et al.*, 1996, Progr. Neurobiol., 48, 1, and references therein).

2.2.3. EPILEPSY

Epilepsy, as a term, is broadly used to describe a group of disorders that are characterized by transient, recurrent, spontaneous paroxysms of a hyperactive brain resulting in seizures. The interictal (between seizures) state of the epileptogenic cortex displays brief, high-amplitude electrical impulses. Seizures are characterized by loss of inhibitory signals so as to foster hyperexcitability and hyperactivity.

2.3. APOPTOSIS AND INJURY FROM RADIATION OR CHEMOTHERAPY

Apoptosis is a physiological process whereby cells die without swelling, necrosis, or inflammation (J. F. R. Kerr *et al.*, 1972, Brit. J. Cancer, 26, 239). Apoptosis, in a non-pathological context, is considered responsible for the selective deletion of cells during embryogenesis and for homeostasis in continuously renewing tissues (S. Sen, 1992, Biol. Rev., 67, 287). In a pathological context, apoptosis is exhibited by cells in response to irradiation, oxidative stress, and to various chemical stimuli such as glucocorticoids (M. J. Arends, A. H. Wyllie. 1991, Int. Rev. Exper. Pathol., 32, 223). Apoptosis has also been implicated as a process leading to aging (Z. Zakeri and R. A. Lockshin, 1994, Ann. Rev. NY Acad. Sci., 719, 212) and cancer (J. F. R. Kerr *et al.*, 1994, Cancer, 73, 2013). Biophysical and morphological indicators of apoptosis include fragmentation of endonuclear and chromatin DNA (Arends *et al.*, 1990, Amer. J. Pathol., 136, 593) and compaction of cytoplasmic organelles into so-called "apoptotic bodies" (A. H. Wyllie *et al.*, 1980, Int. Rev. Cytol., 68, 251).

2.4. CARCINOGENESIS

A neoplasm, or tumor, is a cellular mass resulting from abnormal uncontrolled cell growth, which may cause swelling on the body surface, and which can be benign or malignant. Benign tumors generally remain localized. Malignant tumors are collectively termed cancers. The term "malignant" generally means that the tumor can invade and destroy neighboring body structures and spread to distant sites to cause death (for review, see Robbins and Angell, 1976, Basic Pathology, 2d Ed., W.B. Saunders Co., Philadelphia, 68).

Effective treatment and prevention of cancer remains a long-felt need, and a major goal of biomedical research.

2.5. AUTOIMMUNE DISEASES

Autoimmune diseases are estimated to affect from 1 to 2 percent of the human population. Such diseases fall into two broad categories: organ-specific, and systemic diseases. Organ-specific autoimmune diseases include myasthenia gravis, Grave's disease, juvenile insulin-dependent diabetes, Addison's disease, and a host of others. Systemic autoimmune diseases include rheumatoid arthritis, systemic lupus erythematosus, scleroderma, rheumatic fever, as well as others. Such diseases result from a breakdown of immune tolerance to self-antigens. Such breakdown may be due to any of one or several

mechanisms: lack of elimination of self-reactive clones, activation of anergic self-reactive cells, or the release of sequestered self-antigens that previously were inaccessible to the immune system.

2.5.1. SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus is characterized by inflammation in many different organ systems, as well as by excessive production of antibodies to nuclear, cytoplasmic and membrane antigens.

2.6. VIRAL DISEASES

Viruses are obligate intracellular parasites whose replication in a host cell is at the molecular level (A. K. Field, 1994, Encyclopedia of Virology, R. G. Webster and A. Granoff, Eds., Academic Press, 42). Viruses are dependent on the host cell energy for metabolism and macromolecular synthesis, particularly with respect to reproduction of their genomes in the hostile environment of the host cell (*id.*). The cycle of viral replication involves attachment of a virion to a host cell, penetration of the host cell, initiation of expression of viral genes, and use of the host cell biosynthetic apparatus to generate viral proteins and nucleic acids and packaging so as to generate and release progeny virions. Antiviral agents for general and specific viral diseases have been proposed that target each of the stages in the viral replication cycle (*id.*).

2.6.1. AIDS AND THE HUMAN IMMUNODEFICIENCY VIRUS

Human immunodeficiency virus (HIV) induces a persistent and progressive infection leading, in the vast majority of cases, to the development of the acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983, Science 220, 868; Gallo et al., 1984, Science 224, 500). There are at least two distinct types of HIV: HIV-1 (Barre-Sinoussi et al., 1983, Science 220, 868; Gallo et al., 1984, Science 224, 500) and HIV-2 (Clavel et al., 1986, Science 233, 343; Guyader et al., 1987, Nature 326, 662). In humans, HIV replication occurs prominently in $CD4^+$ T lymphocyte populations, and HIV infection leads to depletion of this cell type and eventually to immune incompetence, opportunistic infections, neurological dysfunctions, neoplastic growth, and ultimately death.

HIV is a member of the lentivirus family of retroviruses (Teich et al., 1984, RNA Tumor Viruses, Weiss et al., eds., CSH-press, 949). Retroviruses are small enveloped viruses that contain a single-stranded RNA genome, and replicate via a DNA intermediate produced by a virally-encoded reverse transcriptase, an RNA-dependent DNA polymerase

(Varmus, H., 1988, Science 240, 1427). Other retroviruses include, for example, oncogenic viruses such as human T-cell leukemia viruses (HTLV-1,-II,-III), and feline leukemia virus.

The first isolates of HIV were of the HIV-1 subtype; this subtype is now pandemic. HIV-1 infects T lymphocytes, monocyte-macrophages, dendritic cells, and glia within the central nervous system (*e.g.*, microglia, astrocytes) (Gartner *et al.*, 1986, Science 233, 215; Koenig *et al.*, 1986, Science 233, 1089; Pope *et al.*, 1994, Cell 78, 389; Weissman *et al.*, 1995, Proc. Natl. Acad. Sci. USA 92, 826; Schmidtmayerova *et al.* 1996, Proc Natl. Acad. Sci. USA, 93, 700). All these cell types express the CD4 glycoprotein, which serves as a receptor for HIV-1 and HIV-2 (Dalgleish *et al.*, 1984, Nature 312, 763; Klatzmann *et al.*, 1984, Nature 312, 767; Maddon *et al.*, 1986, Cell 47, 333).

HIV, like other enveloped viruses, introduces viral genetic material into the host cell through a viral-envelope mediated fusion of viral and target membranes. HIV-1 infection is mediated through the binding of the virus to the CD4 glycoprotein and other co-receptors. The HIV-1 envelope glycoproteins gp41 (a transmembrane protein) and gp120 (a cell surface protein) direct this binding. gp120 is non-covalently attached to gp41, which is anchored in the viral lipid bilayer. HIV-1 entry is mediated by the high-affinity binding of gp120 to the amino-terminal domain of the CD4 glycoprotein, causing conformational changes in gp120 (McDougal *et al.*, 1986, Science 231, 382; Helseth *et al.*, 1990, J. Virol. 64, 2416; Wain-Hobson, 1996, Nature 384, 117) and subsequent binding of gp120 to co-receptors, such as CXC-CKR4 and CC-CKR5 (Wu *et al.*, 1996, Nature 384, 179; Trkola *et al.*, 1996, Nature, 384, 184; Wain-Hobson, 1996, Nature 384, 117).

Individuals afflicted with AIDS exhibit progressive loss of CD4+ T lymphocytes, the major cell target of the virus (Fauci *et al.*, 1984, Ann. Int. Med., 100, 92) and slow deterioration of the immune system. In consequence, these individuals suffer from a variety of opportunistic infections and certain types of cancers (Levy, 1989, J. Am. Med. Assoc., 261, 2997) that ultimately prove fatal in the vast majority of cases.

HIV infection is pandemic and HIV-associated diseases represent a major world health problem. Although considerable effort is being put into the design of effective compounds, currently no curative anti-retroviral drugs against AIDS exist. In attempts to develop such drugs, several stages of the HIV life cycle have been considered as targets for therapeutic intervention (Mitsuya, H. *et al.*, 1991, FASEB J., 5, 2369-2381). Many viral targets for intervention with HIV life cycle have been suggested, as the prevailing view is that interference with a host cell protein would have deleterious side effects. For example, virally encoded reverse transcriptase has been one focus of drug development. A number of reverse-transcriptase-targeted drugs, including 2′,3′-dideoxynucleoside analogs such as

AZT, ddI, ddC, and d4T have been developed which have been shown to been active against HIV (Mitsuya, H. *et al.*, 1991, Science, 249, 1533). While beneficial; these nucleoside analogs are not curative, probably due to the rapid appearance of drug resistant HIV mutants (Lander, B. *et al.*, 1989, Science, 243, 1731). In addition, the drugs often cause toxic side effects such as bone marrow suppression, vomiting, and liver function abnormalities.

Attempts are also being made to develop drugs which can inhibit viral entry into the cell, the earliest stage of HIV infection. Here, the focus has thus far been on CD4, the cell surface receptor for HIV. Recombinant soluble CD4, for example, has been shown to inhibit infection of $CD4^+$ T cells by some HIV-1 strains (Smith, D.H. *et al.*, 1987, Science, 238, 1704). Certain primary HIV-1 isolates, however, are relatively less sensitive to inhibition by recombinant CD4 (Daar, E. *et al.*, 1990, Proc. Natl. Acad. Sci. USA, 87, 6574). In addition, recombinant soluble CD4 clinical trials have produced inconclusive results (Schooley, R. *et al.*, 1990, Ann. Int. Med., 112, 247; Kahn, J.O. *et al.*, 1990, Ann. Int. Med., 112, 254; Yarchoan, R. *et al.*, 1989, Proc. Vth Int. Conf. on AIDS, p. 564, MCP 137).

The late stages of HIV replication, which involve crucial virus-specific processing of certain viral encoded proteins, have also been suggested as possible anti-HIV drug targets. Late stage processing is dependent on the activity of a viral protease, and drugs are being developed which inhibit this protease (Erickson, J., 1990, Science, 249, 527). The clinical outcome of these candidate drugs is still in question.

Attention is also being given to the development of vaccines for the treatment of HIV infection. The HIV-1 envelope proteins (gp160, gp120, gp41) have been shown to be the major antigens for anti-HIV antibodies present in AIDS patients (Barin, *et al.*, 1985, Science, 228, 1094). Thus far, therefore, these proteins seem to be the most promising candidates to act as antigens for anti-HIV vaccine development. Several groups have begun to use various portions of gp160, gp120, and/or gp41 as immunogenic targets for the host immune system. See for example, Ivanoff, L. *et al.*, U.S. Pat. No. 5,141,867; Saith, G. *et al.*, PCT Publ. No. WO 92/22,654; Shafferman, A., PCT Publ. No. WO 91/09,872; Formoso, C. *et al.*, PCT Publ. No. WO 90/07,119. To this end, vaccines directed against HIV proteins are problematic in that the virus mutates rapidly rendering many of these vaccines ineffective. Clinical results concerning these candidate vaccines, however, still remain far in the future.

Thus, although a great deal of effort is being directed to the design and testing of anti-retroviral drugs, effective, non-toxic treatments are still needed.

2.6.2. AlMV AND ILARVIRUSES

Alfalfa mosaic virus (AlMV) and the related ilarviruses such as tobacco streak virus are economically important pathogens whose coat proteins contain zinc-finger domains. To be infectious, such viruses require genome activation by a few molecules of the coat proteins or nucleic acids coding for them. These viruses are of economic significance because crop infections by them result in losses to alfalfa, tobacco, clover, pea, potato, pepper, tomato celery and prune farmers.

2.6.3. HUMAN PAPILLOMA VIRAL CARCINOGENESIS

Human papilloma viruses (HPV) are DNA viruses, most of which merely cause benign warts. Of the 60 known HPVs, several are known to be involved in invasive cancer of the uterine cervix. One distinguishing feature of such cancer-involved HPVs, is that E7 proteins are known to be expressed in them.

2.6.4. SEMLIKI FOREST VIRUS

Semliki Forest Virus (SFV) is a mosquito-borne pathogen that in humans, can cause arthralgia, rash and fever. Along with Sindbis virus, it is a member of the *Togaviridae Alphavirus* genus. It has been isolated in Africa, India and Southeast Asia, and is primarily found in small wild animals, birds and subhuman primates.

2.6.5. HEPATITIS C

Hepatitis C is the major etiological agent of human parenterally and community-acquired non-A and non-B hepatitis. Chronic hepatitis C is estimated to be carried by 300 million humans worldwide, and is more prevalent in Europe and Japan than either Hepatitis A or B. Protective vaccination is not available for Hepatitis C, and no truly effective treatment is available for the disease.

2.6.6. MEASLES

Measles viral infection in non-immune humans causes fever, cough, conjunctivitis and a rash. Complications of the disease can lead to pneumonia and immunodepression that leads to bacterial invasion of the lungs; occasionally, post-infectious encephalitis may result and can lead to demyelination with risk of mortality or loss of intellectual function. There are no known chemotherapeutics effective against measles infection, although hyperimmune serum globulin can prevent or modify the disease if given within 5 days of exposure.

2.7. DRUG AND ALCOHOL ADDICTION

Drug addiction or dependence involves repeated, compulsive use of a drug in order to receive its chemical rewarding effects or to avoid the punishing effects of drug withdrawal (C. Page and M. J. Curtis, 1997, "Integrated Pharmacology", Mosby International, London, ch. 30; A.J. Roberts and G.F. Koob, 1997, Alcohol Health and Research World, 21, 101-106; J. Blundell, 1991, TiPS, 12, 147-157). All drugs that produce dependence have chemical effects within the brain and provide pleasurable sensations or rewards, such as anti-fatigue, relaxation, or euphoria. Such rewarding sensations (hereinafter "reward system") are due to potentiation of neurotransmitters in the brain; neurotransmitters implicated in this process are γ-aminobutyric acid (hereinafter, "GABA"), dopamine, norepinephrine, acetylcholine, glutamate, endorphin, and serotonin (*id.*).

The diligent search for promising alternative pharmacological and therapeutic approaches to alcohol abuse has been extensively documented in a Symposium of the Nobel Foundation (B. Jansson, H. Rydberg, L. Terenius and B. L. Vallee, *Toward a Molecular Basis of Alcohol*, Birkhäuser Virlag, Basel, 1993). The study of the mode of action of addictive drugs proposes a reward system whose existence has not been proven experimentally. Receptors of GABA, dopamine, glutamate, serotonin, opium, heroin, cocaine, and corticotropin-releasing factor, as well as receptors of other neurotransmitters continued to be identified in large numbers and have been shown to play significant roles in the mode of action of addictive drugs, such as heroin. Both their individual and collective role(s) in the mechanisms of action and pharmacology of addictive drugs have been and remain under expanding, intensive investigation. The speculation that ethanol acts in a manner similar or identical to such drugs has received such wide attention that many now consider this a fact. There is, however, no direct experimental evidence for this assumption (A. Goldstein, *Addiction*, W. H. Freeman and Co., New York, 1994), a disappointment which has both caused much discouragement as well as persistent efforts to re-examine and extend present knowledge (*see* B. L. Vallee, 1997, Proc. Roy. Instit, 68, 1997).

2.8. EBSELEN AS A THERAPEUTIC AGENT

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one is known for the treatment of rheumatism (DE-OS 3027073; U.S. Pat. No. 4,352,799) and for the treatment of oxidative stress (DE-OS 3616923). Ebselen solid drug pharmaceutical preparations are also known (U.S. Pat. No. 5,021,242. None of these disclosures, however, suggest in any

way the use of Ebselen in the control of zinc homeostasis by control of cellular redox state so as to promote release of zinc from MT.

Citation or discussion of a reference hereinabove shall not be construed as an admission that such is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention is directed to therapeutic methods and compositions that control metallothionein mediated zinc transfer.

A method is disclosed for modulating the release of zinc from metallothionein within a cell comprising altering the concentrations of glutathione and glutathione disulfide within a cell.

A method is disclosed of promoting the release of zinc from metallothionein within a cell comprising increasing the concentration of glutathione disulfide within the cell.

A method is disclosed of promoting the release of zinc from metallothionein within a cell comprising increasing the concentration of glutathione disulfide within the cell in the presence of naturally occurring glutathione.

Methods are disclosed of promoting the release of zinc from metallothionein within a cell comprising administering to the cell a zinc-releasing amount of an oxidizing agent. In a non-limiting embodiment, the oxidizing agent is selected from the group consisting of ebselen, selenocystine, selenocystamine, selenoglutathione disulfide, iron(III) chelates, dehydroascorbate, disulfides, including but not limited to glutathione and coenzyme A disulfides and cystamine, FAD, NAD^+, copper (II) chelates, and combinations thereof.

Methods are also disclosed of inhibiting the release of zinc from metallothionein within a cell comprising increasing the concentration of glutathione within the cell.

Methods are also disclosed of inhibiting the release of zinc from metallothionein within a cell comprising administering to the cell an amount of a reducing agent effective to inhibit the release of zinc from metallothionein. In a non-limiting embodiment, the reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

These methods are applicable to cultured cells and cells contained in a host organism, wherein the organism may be a plant or an animal, and where the animal may be a human.

The invention provides for treatment of disorders of cell fate or differentiation by administration of a therapeutic compound of the invention. Such therapeutic compounds include but are not limited to: ebselen, cytochrome c, ascorbate, dehydroascorbate, GSH, GSSG, coenzyme A, cystamine, cysteamine, FAD, $FADH_2$, azurin, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

A method is disclosed for treatment of a pathological condition in which zinc homeostasis is perturbed, where the method involves administration of an effective amount of a compound, where administration of the compound changes the cellular oxidation state to effect an action selected from the group consisting of causing a release of zinc from MT and prevention of a release of zinc from MT.

The disclosed method is applicable to classes of disorders and disorders including but not limited to : Alzheimer's disease; neurodegenerative diseases including Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis; epilepsy; addictions; severe mental illness including depression and schizophrenia; eating disorders including anorexia nervosa, bulimia and obesity; inflammatory diseases including colitis, ileitis, common cold, dermatitis, asthma and endotoxic shock; disorders of the endocrine system including prostate cancer and hypertrophy, diseases of the thyroid, parathyroid, pituitary and thymus, diseases of the ovaries and diseases of the adrenal glands; pathological apoptosis; carcinogenesis; autoimmune diseases including systemic lupus erythematosus; viral diseases including infection by HIV, hepatitis C, measles, papilloma and Semliki Forest virus.

The method also includes co-administration of a cofactor to better target the molecule to which zinc is released. Cofactors disclosed include ATP, GTP and GSH.

3.1. DEFINITIONS

As used herein, the following terms shall have the meanings indicated:

MT = metallothionein;

Tris = tris(hydroxymethyl)aminomethane;

SDH = sorbitol dehydrogenase (EC 1.1.1.14);

apo-SDH = zinc-depleted SDH;

GSH = glutathione;

GSSG = glutathione disulfide;

PAR = 4-(2-pyridylazo)resorcinol.

T = thionein

CPA = carboxypeptidase A

AP = alkaline phosphatase

HQSA = 8-hydroxyquinoline-5-sulfonic acid

DTNB = 5,5'-dithiobis-(2-nitrobenzoic acid)

EDTA = ethylenediaminetetraacetic acid

zincon = 2-carboxy-2'-hydroxy-5'-sulfoformazyl-benzoic acid

Hepes = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

DsbA = protein disulfide isomerase from *E. coli*

HIV-1 = human immunodeficiency virus type I

NCP = nucleocapsid protein

As used herein, the term compound means any molecule, salt, metal, or any other combination of one or more atoms, including but not limited to covalently bonded molecules, ionic materials, metallic materials, crystalline materials, atoms or molecules or ions in solution, atoms or molecules or ions in the gas phases, and combinations of any of the preceding.

As used herein, the term chelate refers to a complex between one or more metal atoms and one or more ligands where the bonds between the metal and ligand may include any coordinative, dative, ionic, Lewis-basic or covalent interaction, and where at least one ligand occupies at least two coordination sites on at least one metal atom. Examples of ligands forming chelates include but are not limited to nitrogen and oxygen macrocycles such as porphines, chorines, porphyrazines and crown ethers, β-keto carbonyl anionic compounds such as acetylacetonate, multi-dentate amines such as ethylene diamine, bipyridines, and phenanthrolines, polycarboxylate and mixed carboxylate/amine ligands such as EDTA, cyclopentadienyl anion, α-diimines (1,4-diaza-1,3-butadiene derivatives), multidentate phosphines such as diphos and diars, as well as other multi-dentate ligands well known to those of ordinary skill in coordination chemistry; any such ligand may be substituted or unsubstituted, and may be part of a larger molecule such as a polypeptide or protein, and may be immobilized, for example, on a non-polymeric or polymeric support (non-limiting examples of such supports are functionalized polystyrene or other polymeric beads, fibers, sheets, etc., functionalized cellulosic materials such as paper) so as to permit easy introduction of the chelate to a specific site and later removal.

4. DESCRIPTION OF THE FIGURES

Figure 1. Kinetics of the reconstitution of apo-SDH with free zinc (□) and MT-1 (■). Free zinc (1.7 μM) or MT (0.24 μM) (molar ratio between zinc and apo-SDH of 1.0) was incubated with apo-SDH (1.7 μM) in 0.2 M Tris-HCl, pH 7.4. Aliquots (10 μl) were periodically withdrawn from this mixture and assayed for enzymatic activity. Reactivation is expressed as the activity of native SDH recovered.

Figure 2. Kinetics of the reconstitution of apo-SDH (1.7 μM) with 1.7 μM MT-1 (■) and 11.9 μM carbonic anhydrase (□). Conditions were as described in the legend of Figure 1.

Figure 3. Reconstitution of apo-SDH with free zinc (A) and MT-1 (B). Free zinc or MT-1 was incubated with apo-SDH (1.7 μM) under different ratios of zinc to apo-SDH monomers in 0.2 M Tris-HCl, pH 7.4 for 30 min (or 60 min for MT-1). Aliquots (10 μl) were withdrawn from this mixture and assayed for enzymatic activity.

Figure 4. Kinetics of the reconstitution of apo-SDH with MT-1 in the presence of GSSG and/or GSH. MT (0.24 μM) (molar ratio between zinc and apo-SDH of 1.0) was incubated with apo-SDH (1.7 μM) in 0.2 M Tris-HCl, pH 7.4 in the presence of GSSG and/or GSH. (■) in the absence of GSH and GSSG; (♦) in the presence of 3 mM GSSG; (★) in the presence of 3 mM GSSG and 1.5 mM GSH; (□, dashed line) in the presence of 10 μM GSH. Aliquots (10 μl) were periodically withdrawn from the mixtures and assayed for enzymatic activity.

Figure 5. Dependence of the reactivation of apo-SDH on (A) the concentration of GSH at a fixed concentration of 3 mM GSSG, and (B) the concentration of GSSG at a fixed concentration of 1.5 mM GSH. MT (0.24 μM) was incubated with apo-SDH (1.7 μM) in 0.2 M Tris-HCl, pH 7.4 for 60 min at the indicated concentrations of GSH and GSSG. Aliquots (10 μl) were then withdrawn from the mixtures and assayed for enzymatic activity.

Figure 6. Radiochromatograms of ^{65}Zn-MT-2 with apo-SDH in the absence or presence of GSH and GSSG. MT-2 was incubated with apo-SDH (molar ratio between zinc and apo-SDH of 1.0) for 60 min and then the mixture was analyzed by radiochromatography as described under Materials and Methods. A, in the absence of GSH and GSSG; B, [GSH]=1.5 mM, [GSSG]=3 mM. For the determination of the number of zinc atoms transferred, the radiochromatograms were integrated from fractions 14-17. (■) MT control; (□) MT after the reaction with apo-SDH.

Figure 7. Inhibition of zinc transfer from MT-2 by GSH. MT (1.3 μM) was incubated with PAR (100 μM) in 0.2 M Tris-HCl, pH 7.4 in the presence of increasing amounts of GSH and the reaction was followed by measuring the increase of absorbance at 500 nm of the zinc-PAR complex for 60 min. The number of zinc atoms released from MT was calculated on the basis of the formation of $Zn(PAR)_2$ ($\epsilon_{500} = 61{,}500\ cm^{-1}M^{-1}$). Inhibition is expressed as the decrease in the amount of zinc transferred. The dotted line is based on a simple model with one GSH binding site and a dissociation constant of 56 μM.

Figure 8. Concentration dependence of reconstitution of apo-AP with Zn_7MT-2 (-♦-) and Cd_5Zn_2MT-2 (-■-). Apo-AP, 0.5 μM, was incubated with various concentrations of Zn_7MT-2 and Cd_5Zn_2MT-2 in 10 mM Tris, pH 8.0. Aliquots were taken after two hours and assayed spectrophotometrically for enzymatic activity.

Figure 9. Reconstitution of apo-AP with Zn_7MT-2 in the presence of oxidizing agents. Apo-AP, 0.5 μM, was incubated with Zn_7MT-2, 0.29 μM, and various concentrations of DTNB (-♦-) and GSSG (-■-) in 10 mM Tris, pH 8.0. Aliquots were taken after two hours and assayed spectrophotometrically for enzymatic activity. 100 % AP activity corresponds to reactivated apo-AP in the absence of oxidizing agents under otherwise identical conditions.

Figure 10. Concentration dependence of reconstitution of apo-CPA with Zn_7MT-2. Apo-CPA, 2 μM, was incubated with various concentrations of Zn_7MT-2 in 20 mM Hepes/100 mM NaCl, pH 7.5. Measurements were taken after 30 min.

Figure 11. Reaction of MT-2 with PAR in the presence of selenite and selenocystamine. MT-2, 0.5 μM, was incubated with PAR, 100 μM, in the absence (-●-) and presence of 50 μM sodium selenite (-♦-) and 50 μM selenocystamine (-■-).

Figure 12. Time dependence of the inactivation of AP by T. AP, 0.5 μM, was incubated with T, 1 μM, in 1 M Tris, pH 8.0. Aliquots were taken at defined time intervals and assayed spectrophotometrically for enzymatic activity.

Figure 13. Effect of Zn(II) on the reaction of T with DTNB. T, 0.5 μM, was incubated with various concentrations of zinc sulfate in 20 mM Hepes, pH 7.5 for 30 s and DTNB added to

a final concentration of 50 μM. Relative reactivity of thiols was determined from measurements taken after 30 s.

Figure 14. Zinc release from MT by ebselen. (A) Time dependence of zinc transfer from MT-2 (0.5 μM) to PAR (100 μM) in the absence (-■-) and presence (-♦-) of ebselen (10 μM in methanol) in 20 mM Hepes, pH 7.5 at 25 °C. (B) Zinc transfer from MT (0.2 μM) to the apoform of carboxypeptidase A (1 μM) as a function of the concentration of ebselen under the same conditions as in (A).

Figure 15. Kinetics of zinc release from MT-2 induced by the glutathione redox couple. MT (1.3 μM) was incubated with PAR (100 μM) in 0.2 M Tris-HCl, pH 7.4, in the absence of glutathione, squares; in the presence of 1.5 mM GSH and 3 mM GSSG, circles. Zinc release was followed by measuring the formation of $Zn(PAR)_2$ at 500 nm.

Figure 16. Kinetics of zinc release from MT-2 by horse heart cytochrome c. MT (0.43 μM) was incubated with PAR (60 μM) in 0.2 M Tris-HCl, pH 7.4, squares; in the presence of 100 μM cytochrome c, circles. Zinc release was measured as described in the legend of figure 1.

Figure 17. Kinetics of zinc release from MT by dithiodipyridine and concomitant sulfhydryl oxidation. MT (0.5 μM) was dissolved in degassed, 20 mM Hepes, pH 7.5 and incubated with 100 μM zincon (to measure zinc release, right ordinate, circles) and 50 μM dithiodipyridine (to measure thiol oxidation, left ordinate, squares).

Figure 18. Kinetics of the reaction of MT-2 with protein disulfide isomerase (DsbA). MT (0.75 μM) was incubated with the indicated amounts of DsbA and PAR (90 μM) in 40 mM Hepes, pH 7.4; MT control, squares; one equivalent of DsbA, circles; two equivalents of DsbA, diamonds; three equivalents of DsbA, triangles.

Figure 19. Kinetics of the reaction of ebselen with MT followed by UV-VIS spectroscopy. (A) MT-2 (0.5 μM) and ebselen (10 μM) in 20 mM Hepes, pH 7.5 at 25 °C between 0 and 24 sec. (B) Difference spectra calculated from the spectra shown in (A).

Figure 20. Stopped-flow kinetics of the reactions between ebselen and MT (A) and ebselen and glutathione (B). (Conditions are given in the legends on the printouts.)

Figure 21. Titration of MT with Ebselen. MT-2 (0.5 μM) was titrated with increasing amounts of ebselen and zinc release determined with PAR.

Figure 22. Zinc release (left ordinate, -♦-) from yeast alcohol dehydrogenase by ebselen and concomitant decrease of enzymatic activity (right ordinate, -■-) . Yeast alcohol dehydrogenase (0.5 μM) was reacted with increasing aliquots of ebselen in 20 mM Hepes (nitrogen-saturated), pH 7.5 in the presence of the indicator PAR (100 μM). At completion of the reaction (20 min), endpoint readings were taken to calculate zinc transfer from the formation of a $Zn(PAR)_2$ complex (ϵ_{500} = 65,000 $M^{-1}cm^{-1}$). Extent of zinc transfer is based on the zinc analysis for yeast ADH

Figure 23. Catalytic activity of selenocystine. 0.5 μM MT (rabbit) was added to a nitrogen purged solution of 100 μM PAR in 20 mM Hepes, pH 7.5 in a quartz cuvette. ^{t}BuOOH (10 mM stock in water) was added to a final concentration of 100 μM and catalytic amounts of DL-selenocystine (1 mM stock in 0.01 M HCl, pH 2.0) were added. The cuvette was closed by laboratory film and the solution was mixed by gently turning the closed cuvette. The cuvette was inserted into a Cary 1E spectrophotometer (Varian) thermostated 6 x 6 cell holder (25°C) and changes in absorption at 500 nm were recorded. Control experiments were recorded at the same time in the same cell holder. -♦- no oxidant; -■- 50 nM DL-selenocystine; -Δ- 100 μM ^{t}BuOOH; -●- 50 nM DL-selenocystine + 100 μM ^{t}BuOOH. ^{t}BuOOH and DL-selenocystine were from Sigma (St. Louis). PAR was from Fluka.

Figure 24. Zinc transfer from human Zn_7-MT to apo-SDH in the presence of ATP (■), AMPPNP (♦) and ADP (★), and in the absence of nucleotides (□). Apo-SDH (1.7 μM) was incubated with 0.24 μM MT in 0.2 M Tris-HCl, pH 7.4 at 22.5°C in the absence or presence of nucleotide (1 mM). Aliquots were withdrawn periodically and assayed for enzymatic activity.

Figure 25. Electronic absorption difference spectra of binding of ATP to MT. The difference spectra of reactions of human MT-2 (2.5 μM) with different concentrations of ATP were recorded on a Cary model 1E high performance UV-Vis spectrophotometer (Varian) using Tandem cuvets as desribed in Material and Methods.

Figure 26. Spectrophotometric detection of ATP binding to rabbit Cd,Zn MT-2 by the Hummel-Dreyer method. MT (142 μM MT) was dissolved in 50 mM Hepes, pH 7.4

containing ATP (100 μM), loaded onto a Sephadex G-25 column, and eluted with the same buffer. Identical results were obtained when human Zn_7-MT was employed.

Figure 27. Scatchard plot of ATP binding to rabbit Cd,Zn MT-2. Conditions as described in Figure 26.

Figure 28. Quenching of fluorescent ε-ATP by rabbit Cd,Zn MT-2. ε-ATP (20 μM) was dissolved in 0.2 M Tris-HCl, pH 7.4. Aliquots of MT were added and emission spectra recorded with excitation at 274 nm (5 nm slits).

Figure 29. ATP binding to MT in the presence of glutathione. ATP (100 μM) was incubated with rabbit Cd,Zn MT-2 (142 μM) in 50 mM Hepes, pH 7.4 in the absence of glutathione or in the presence of either GSH (120 μM) or GSSG (500 μM). Binding was evaluated as described in Figure 26.

Figure 30A,B. Reactivity of thiols of MT in the absence and presence of ATP. Human MT-2 (10 μM) was incubated with (■) or without (□) ATP (1 mM) and DTNB (4 μM) in 0.2 M Tris, pH 7.4 and the reaction followed spectrophotometrically.

Figure 31. The effect of ATP on elution behavior of MT in gel filtration. Cd,Zn MT-2 (0.5 mg) was dissolved in 50 mM Hepes, pH 7.4, 10 mM NaCl in the presence or absence of 1 mM ATP, and subjected to gel filtration on a Sephadex G-75 column (100 x 1 cm) at 25 °C an a flow rate of 6.7 mL/h Elution of MT (-■-); elution of MT/ATP complex (-□-).

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to therapeutic methods and compositions that alter zinc metabolism by means of an increase or decrease in zinc transfer between metallothionein and cellular and extracellular zinc acceptors. By administration of a therapeutic compound of the invention, the invention provides for treatment pathological conditions in which zinc is perturbed including those delineated in Table 1, below. Such therapeutic compounds (termed herein "compounds") include but are not limited to: ebselen, cytochrome c, ascorbate and dehydroascorbate, GSH and GSSG, coenzyme A, cystamine and cysteamine, FAD and $FADH_2$, azurin, vitamin E, and pro-GSH agents such as N-acetyl-L-cysteine and α-lipoate. Preferred among these compounds are ebselen, ascorbate and dehydroascorbate,

GSH and GSSG, cystamine and cysteamine, azurin, vitamin E, and pro-GSH agents such as N-acetyl-L-cysteine and α-lipoate. The compound most preferred is ebselen.

Table 1. Conditions susceptible to treatment with compounds of the invention.

Condition	Non-limiting Examples
Disorders of the central nervous system	Alzheimer's disease Neurodegenerative diseases including Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Lou Gehrig's disease), Epilepsy Addictions Severe mental illness including depression and schizophrenia Eating disorders including anorexia nervosa, bulimia and obesity
Inflammatory diseases	Colitis Ileitis Common cold Dermatitis Asthma Endotoxic shock
Disorders of the endocrine system	Prostate cancer or hypertrophy Disorders of the thyroid or parathyroid Disorders of the pituitary Disorders of the thymus Disorders of the ovaries Disorders of the adrenal gland
Apoptosis and injury from radiation or chemotherapy	Pathological apoptosis
Carcinogenesis	
Autoimmune diseases	Systemic Lupus Erythematosus

Table 1. Conditions susceptible to treatment with compounds of the invention.

Condition	Non-limiting Examples
Viral diseases	HIV/AIDS Hepatitis C Measles Papilloma Semliki Forest Virus
Plant diseases	Alfalfa mosaic virus Ilarviruses

Zinc in MT is bound extremely tightly (K_D about 10^{-13} M). Zinc, in contrast to copper or iron, is redox-inert. Hence, the properties of zinc complexes are not altered by valence changes of the central atom in a manner akin to those in iron or copper complexes. However, the redox state of the sulfur ligands can be changed. The cluster structure of MT provides the chemical basis by which the cysteine ligands can induce oxidoreductive properties. Hence, the cluster structure focuses on the significance of zinc/cysteine thiolate coordination in MT as the critical arrangement for zinc in this molecule to render the complex oxidoreductive. This allows for thermodynamic stability of zinc in MT while permitting zinc to retain kinetic lability. This is demonstrated experimentally by the ensuing facile and fast zinc exchange between MT and the apoforms of zinc proteins. Thus kinetic lability can be introduced into the zinc-thiolate clusters by oxidoreduction of the sulfur donor atoms. Indeed, the data presented in the Experimental Results section below demonstrate that a large number of agents oxidize the thiolate ligands even while these ligand remain coordinated to zinc. In point of fact, the reducing potential of MT remains sufficiently low to allow oxidation by physiological oxidants such as disulfides or selenium compounds. Thus, the existence of these clusters in MT is unique to biology and has a role that could not have been predicted from either known zinc or sulfur chemistry. These clusters embody a completely new utilization of Zn-S chemistry in conferring dynamic behavior on complexes with inherently high stability. This then establishes a hitherto unknown mechanism that has a role in cellular zinc distribution.

Further insight into how MT may act as an agent that controls zinc distribution is found with zinc release and transfer to the apoform of a zinc enzyme sorbitol dehydrogenase. This release and transfer are modulated by both reduced glutathione (GSH) and oxidized glutathione (GSSG). GSH alone inhibits zinc release in the absence of GSSG,

indicating that the MT is stabilized at relatively high cellular GSH concentrations. The presence of GSSG (or any other oxidizing agent) results in release of zinc that is synergistically increased by GSH. Since zinc release from MT depends on both GSH and GSSG in a concentration-dependent manner, the supply of zinc from MT is redox-regulated in a concentration-dependent manner, i.e., zinc distribution is coupled both to energy metabolism and the cellular redox state. *In vitro*, MT not only acts as a zinc donor, but in the form of thionein, acts also as a zinc acceptor. These reactions are not strictly reversible and depend on additional factors and conditions, thus adding another level of regulation to effect kinetic partitioning of zinc.

Zinc plays a central role in cellular metabolism, and zinc performs regulatory functions. Zinc is a modulator of synaptic transmission, which is an extracellular regulatory function. It is quite likely that zinc also is an intracellular regulator that operates at concentrations different from those of calcium and hence coordinates different sets of biochemical processes.

The present invention is directed to a novel and surprising exploitation of the heretofore unknown relationship between cellular redox state and MT-mediated zinc transfer. The sections below discuss the relationship of zinc to certain disease states. It must be emphasized that nowhere in the literature is there a suggestion of treatment or prevention of disease by regulation of zinc homeostasis through regulation of the MT redox state. The present invention is directed toward the up-regulation and down-regulation of intra- and extracellular zinc by specific regulation of the redox state of metallothionein. Increase in the oxidation potential of the environment surrounding MT will favor release of zinc so as to increase the amount of zinc available for biochemical processes, while decrease in the oxidation potential of the MT environment will favor retention of zinc by MT so as to decrease the amount of zinc available for biochemical processes.

Other factors are involved in directing MT to transfer zinc to a particular target recipient. For example, zinc signatures are available for zinc transfer to particular apoproteins and enzymes (B. Vallee and D. S. Auld, 1993, Acc. Chem. Res.,26, 543). Where zinc-containing active sites are not at the apoprotein surface, a specific interaction between MT and the apoprotein determines the target. Such interactions are due in some cases to ATP, GTP or GSH binding to the surface of MT which binding provides a recognition factor for the target protein. In the case of Zn finger proteins, the active sites are often at the surface, and the recognition may involve other +influences than binding of GSH and/or ATP or GTP.

5.1. METHODS APPLICABLE TO ZINC DEFICIENT SUBJECTS

In cases where a subject is in need of treatment involving release of zinc from MT and is also suffering from a systemic or localized zinc deficiency, the methods described below preferably further comprise prior administration or coadministration of a zinc salt, MT, or a combination of a zinc salt and MT. Such prior or coadministration is preferable because release of zinc from MT requires administration of oxidizing compounds, and subjects suffering from a zinc deficiency typically are already undergoing oxidative stress. Such prior or coadministration of zinc salts and/or MT will reduce the oxidative stress in a zinc-deficient subject, and thereby permit administration of oxidizing compounds without unduly contributing to further oxidative stress in the zinc-deficient subject.

5.2. DISORDERS OF THE CENTRAL NERVOUS SYSTEM

Zinc is concentrated in vesicles of the pre-synaptic terminals of certain glutamatergic neurons and is released during high-frequency neuronal firing, and metallothionein-III ("MT-III") is abundant in these neurons (J. C. Erikson *et al.*, 1997, J. Neuroscience, 17, 1271-1281, and references therein). Zinc is known to have a wide variety of neuromodulatory functions, and to be involved in the pathophysiology of several neurological disorders (*id.*). Zinc has been found to be neurotoxic, and its intracellular accumulation may contribute to nerve death in seizure disorders (*id.*).

In one embodiment of the present invention, decrease in the oxidation potential of the MT environment by addition of reducing agents prevents release of zinc by MT and thereby reduces intracellular accumulation of zinc. Thus in one embodiment, the invention provides a method of treating a subject having a disease of the central nervous system comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the disease of the central nervous system.

5.2.1. ALZHEIMER'S DISEASE

While the relationship between zinc and the growth of neurofibrillary tangles is not altogether clear, the relationship between zinc and the growth of senile plaques is clear. A major component of senile plaques in the AD brain is $A\beta_{1-40}$, a 40 residue polypeptide whose precipitation from cerebrospinal fluid leads to the formation of $A\beta$ amyloid plaque. $A\beta_{1-40}$ has been shown to bind zinc saturably, and the Zn-$A\beta_{1-40}$ complex has been shown to precipitate out of cerebrospinal fluid at physiological zinc concentrations

(A. Bush *et al.*, 1994, Science, 256, 1464-1467; X. Huang *et al.*, 1997, J. Biol. Chem., 272, 26464-26470).

In one embodiment of the present invention, prevention of accumulation or further accumulation of the amyloid plaques characteristic of Alzheimer's disease is effectuated by treatment with reducing and shuttle agent compounds of the present invention. Particularly preferred among the compounds of the current invention for this embodiment are certain thiols, and citrate and dehydroascorbate derivatives. Thus in one embodiment, the invention provides a method of treating a subject having Alzheimer's disease comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the Alzheimer's disease.

5.2.2. NEURODEGENERATIVE DISEASES

Various neurodegenerative disorders are associated with free radicals (J. A. Knight, 1997, Annal. Clin. Lab. Sci., 27, 11). For example, amyotrophic lateral sclerosis (Lou Gehrig's disease), Parkinson's disease, Down's syndrome and multiple sclerosis have all been found to be associated with excess free radicals, particularly oxygen-based free radicals (*id.*).

Prevention of release of zinc from MT mitigates the damage from such oxygen-based free radicals. Without committing ourselves to a particular theory or mechanism, such oxygen-based free radicals result in release of inter- and intracellular zinc from MT. Influx of zinc has been implicated in the mechanism for neuronal death following ischaemic insult (J-Y Koh *et al.*, 1996, Science, 272, 1013). Chelation of extracellular Zn^{2+} by calcium ethylenediaminetetraacetic acid blocks the death of rat hippocampal neurons following ischaemic insult (*id.*) leading to the conclusion that Zn^{2+} toxicity is at least in part responsible for neuronal death following ischaemic events (*id.*).

In one embodiment of the present invention, a compound is administered to a cell so as to maintain a reducing potential in the MT environment. The maintenance of MT in its reduced state prevents the release of zinc, thereby preventing damage from zinc influx. Thus in one embodiment, the invention provides a method of treating a subject having a neurodegenerative disease comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the neurodegenerative disease. The neurodegenerative disease may be selected from the group consisting of Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, etc.

5.2.3. PARKINSON'S DISEASE

Oxidative stress is believed to be a cause of Parkinson's disease. (*id.*) The degeneration of dopaminergic neurons results in increased metabolism of dopamine and a resultant increase in hydroxyl radicals. This same effect can be produced by 6-hydroxydopamine and related substances, the introduction of which can cause parkinsonism (*id.*). Further evidence for the involvement of oxidative stress is found with the correlation between the extent of neuronal loss in Parkinson's patients and a depletion in glutathione (*id.*).

Promotion of release of zinc from MT generally mitigates damage from oxidative stress because available zinc competes with iron and copper species for reactive oxygen species and by protection of cellular thiols. Without committing ourselves to a particular theory or mechanism, it is believed that release of inter- and intracellular zinc from MT prevents damage due to oxidizing species, and treatment with oxidizing compounds promotes release of zinc from MT and thereby prevents neuronal death associated with Parkinson's disease. Thus, in one embodiment, the invention provides a method of treating a subject having Parkinson's disease comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the Parkinson's disease.

5.2.4. EPILEPSY

Zinc has been found to diminish synaptic inhibition by γ-aminobutyric acid ("GABA") in a rat model for epilepsy. One condition associated with epilepsy is hyperexcitability of synapses in the mammalian forebrain (P. A. Schwartzkroin, Ed., 1993, Epilepsy: Models, Mechanisms, and Concepts, Cambridge Univ. Press). This hyperexcitability is favored by a reduction in synaptic inhibition, and synaptic inhibition is primarily mediated by GABA.

Zinc is associated with synaptic inhibition in epilepsy. The dentate gyrus of several experimental models, as well as of humans suffering from temporal lobe epilepsy ("TLE") exhibits a distinctive aberrant sprouting of mossy fibers (S. Otis, *et al.*, 1994, Proc. Natl. Acad. Sci. U.S.A., 91, 7698). For example, the Wistar rat kindling model for epilepsy has enhanced functional inhibition of GABA (*id.*). The mossy fibers of this and other models, as well as those of humans, are loaded with Zn^{2+} that can be released on stimulation (C. J. Fredrickson and D. W. Moncrief, 1994, Biol. Signals, 3, 127). Furthermore, Zn^{2+} has been shown to inhibit certain types of GABA receptors, particularly during early development (T. G. Smart *et al.*, 1994, Prog. Neurobiol., 42, 393). Zn^{2+} has been shown

reversibly to antagonize epileptic granule cell $GABA_A$ receptors so as to block inhibitory post-synaptic currents (E. H. Buhl, 1996, Science, 271, 369-73).

MT-III is a growth-inhibitory factor and is expressed by zinc-containing neurons. MT-III has been suggested to be involved in seizure disorders (J. C. Erickson *et al.*, 1997, J. Neurosci., 17, 1271), and MT-III knockout mice have been found to be more susceptible to seizures induced by treatment with kainic acid (*id.*).

In one embodiment of the present invention, reducing compounds are administered to patients suffering from epilepsy so as to prevent the release of zinc from MT. Prevention of release of zinc from MT reduces the hyperexcitability characteristic of epileptic pathology, and thereby mitigates epileptic symptoms. Thus, in one embodiment, the invention provides a method of treating an epileptic subject comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the epilepsy.

5.2.5. ADDICTIONS

Zinc metabolism has been related to alcoholism. For example, the concentration of zinc has been shown to be altered in the tissues of alcoholics (M. Ebadi *et al.*, 1995, Neurochem. Int., 27, 1-22).

In one embodiment of the present invention, oxidizing compounds are administered along with disulfiram so as to promote release of zinc from MT. Without committing to any particular theory or mechanism, it is believed that such zinc release from MT in conjunction with disulfiram leads to improved anti-drinking effects as compared to disulfiram alone.

Zinc metabolism is also related to withdrawal from cocaine (D. J. Ennulat and B. M. Cohen, 1997, Brain Res. Mol. Brain. Res., 49, 299-302). Cocaine treated rats have been shown to have repressed levels of mRNA coding for a zinc-finger protein transcriptional regulator. After 2 days without cocaine, the level of this mRNA was found to have returned to normal. At the same time mRNA for a different zinc-finger protein transcriptional regulator was found to be induced by cocaine treatment (*id.*).

In one embodiment of the present invention, oxidative compounds are administered to maintain high levels of available zinc. Without committing to any particular theory or mechanism of action, it is believed that such high levels of available zinc interferes with the biochemical reward system (C. Page and M.J. Curtis, 1997, "Integrated Pharmacology", Mosby International, London, ch. 30; A.J. Roberts and G.F. Koob, 1997, Alcohol Health and Research World, 21, 101-106; J. Blundell, 1991, TiPS, 12,

147-157) leading to reduced desire for cocaine. Thus, in one embodiment, the invention provides a method of treating a subject having a drug or alcohol addiction comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the addiction.

5.2.6. SEVERE MENTAL ILLNESSES

The concentration of zinc in the brains of schizophrenics is altered (M. Ebadi *et al.*, 1995, Neurochem. Int., 27, 1-22). Furthermore, patients with chronic major depression, particularly treatment-resistant depression have been found to have increased levels of interleukin 6 that correlate with decreased levels of serum zinc (M. Maes *et al.*, 1997, Cytokine, 9, 853-8), and decreased levels of tryptophan that correlates with decreased levels of zinc (M. Maes *et al.*, 1997, Eur. Arch. Psych. Clin. Neurosci., 247, 154-61), suggesting that cell-mediated immune response may be implicated in such depressions.

In one embodiment, the invention provides method of treating a subject with severe mental illness comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the severe mental illness.

5.2.7. EATING DISORDERS

Zinc is associated with energy metabolism and has been implicated in several eating disorders. For example, zinc is believed to be associated with the energy homeostasis of obesity via its interaction with dietary fat consumption (M. D. Chen *et al.*, 1996, Biol. Trace. Elem. Res., 52, 125-32). Support for this belief is found, for example, in the correlation between hair zinc concentration and obesity (S. K. Taneja *et al.*, 1996, Experientia, 52, 31-3).

In one embodiment, the invention provides a method for treating a subject having an eating disorder selected from the group consisting of anorexia nervosa, bulimia and obesity by administering to the subject an amount of a compound effective to treat said condition, wherein the compound changes the cellular oxidation state to effect an action selected from the group consisting of causing a release of zinc from metallothionein and preventing release of zinc from metallothionein.

In another embodiment, the invention provides A method of treating a subject with anorexia nervosa comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the anorexia nervosa.

In another embodiment, the invention provides a method of treating an obese subject comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the obesity.

5.3. INFLAMMATORY DISEASES

Various disorders exhibiting inflammatory response have been shown to depend on zinc or to respond to zinc therapy. Nowhere, however, has there been a suggestion of the novel therapies of the present invention where control of zinc at the biomolecular level is effectuated by control of the MT redox state. Free ionic zinc in saliva has been shown to decrease the duration and severity of the common cold (S. G. Novick *et al.*, 1997, Med. Hypoth., 49, 347-57, and references therein). Such decreases are proposed to be due to zinc blocking the docking of human rhinovirus to intercellular adhesion molecule 1 ("ICAM-1"); because docking to ICAM-1 is also necessary for initiation of the inflammatory response, zinc ion is believed to be an important inhibitory factor to inflammation, as well (*id.*). Zinc has also been shown to enhance the expression of ICAM-1 in cells actively involved in inflammatory response (S. Martinotti *et al.*, 1995, Biochem. Biophys. Acta, 1261, 107-14).

In one embodiment, the invention provides a method of treating a subject with an inflammatory disease comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the inflammatory disease.

Colitis and Crohn's disease reveal decreased zinc superoxide dismutase activity (L. Lih-Brody *et al.*, 1996, Digest. Dis. Sci., 41, 2078-86) and colitis has been experimentally successfully treated in rats using a zinc chelate compound (T. Yoshikawa *et al.*, 1997, Digestion, 58, 464-8). Lung epithelial tissue in asthmatics, furthermore, has been shown to have reduced zinc-specific activity, perhaps due to inflammatory responses.

In one embodiment, the invention provides a method for treating a subject with colitis or Crohn's disease comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the inflammatory disease.

In another embodiment of the present invention, oxidizing compounds are administered by inhalation so as to treat asthma.

5.4. DISORDERS OF THE ENDOCRINE SYSTEM

Prostate glands of most mammals are known to contain among the highest levels of zinc of any tissues. In benign prostate hyperplasia, zinc levels are increased over normal prostate levels and are known to inhibit androgen metabolism (S. Dutkiewicz, 1995, Materia Medica Polona, 27, 15). Antioxidant enzyme expression, furthermore, is reduced in malignant (neoplastic) prostate glands (A. M. Baker *et al.*, 1997, Prostate, 32, 229).

In one embodiment, the invention provides a method of treating a subject suffering from a disorder of the endocrine system comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the disorder of the endocrine system. Disorders of the endocrine system that may be so treated include but are not limited to prostate cancer, prostate hypertrophy, a disorder of the thyroid, a disorder of the parathyroid, a disorder of the ovaries, and a disorder of the adrenal gland.

In one embodiment of the present invention, reducing compounds are supplied to hyperplastic or neoplastic prostate glands so as to reduce release of zinc from MT.

5.5. APOPTOSIS AND INJURY FROM RADIATION OR CHEMOTHERAPY

Zinc is believed to play a role in pathological apoptosis. Where mice or rats have been fed a zinc-deficient diet, "massive" increase in apoptotic bodies has been observed in mucosal cells of their small intestines (M. Elmes, 1977, J. Pathol., 123, 219-23), and thymic atrophy and defective T helper cell function have also been observed (P. J. Fraker *et al.*, 1977, J. Nutr., 107, 1889-95). Furthermore, zinc-deprived mice also have been found to exhibit skin lesions and stunted growth that are linked to apoptosis (G. Fernandes *et al.*, 1979, Proc. Natl. Acad. Sci., 76, 457-61).

While treatment with zinc salts has been shown to inhibit apoptosis, there has never before been even a suggestion of the present regulation of MT redox state so as to regulate cellular and extracellular zinc availability. In cultured cells, addition of zinc salts to the culture medium has been shown to prevent cells from undergoing apoptosis. For example, following γ-irradiation (J. J. Cohen and R. C. Duke, 1984, J. Immunol., 132, 38-42; P. Beletsky *et al.*, 1989, Gen. Physiol. Biophys., 8, 381-98) or exposure to methasone (K. S. Sellins and J. J. Cohen, 1987, J. Immunol., 139, 3199-3206), murine thymocytes undergo DNA or chromatin degradation that is prevented by addition of zinc salts to the culture medium. Furthermore, *in vivo* protection by zinc against apoptosis has been seen in mice, where pretreatment with zinc acetate three days prior to injection of *S. typhimurium* lipopolysaccharide reduced internucleosomal cleavage of thymic DNA as compared to non-

pretreated mice so injected (D. J. Thomas and T. C. Caffrey, 1991, Toxicology, 68, 327-37). These and various other studies have shown that "a chelatable pool of intra-cellular Zn^{2+} influences apoptosis, since influx of Zn^{2+} prevented apoptosis, while chelation of Zn^{2+} induced apoptosis" (F. W. Sunderman Jr., 1995, Annal. Clinic. Lab. Sci., 25, 134-42).

In one embodiment of the present invention, oxidizing compounds are administered to effect release of zinc from MT in order to combat pathological apoptosis. Thus in one embodiment, the invention provides a method of treating a subject with pathological apoptosis comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the pathological apoptosis.

5.6. CARCINOGENESIS

In one embodiment, a compound of the invention is administered to treat a cancerous condition, or to prevent progression from a pre-neoplastic or non-malignant state (*e.g.*, metaplastic condition) into a neoplastic or a malignant state.

In another embodiment of the present invention, oxidizing compounds are administered so as to release zinc from MT at the site of a cancerous condition and thereby induce apoptosis of selected cells. Thus, in one embodiment, the invention provides a method of treating a subject with cancer, said method comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for inducing apoptosis of the cells of the cancer.

In another embodiment of the present invention, reducing compounds are administered so as to inhibit release of zinc from MT, thereby limiting the bioavailability of zinc as would be required for cellular proliferation, and thus reducing the proliferation of selected cells. Thus, in one embodiment, the invention provides a method of treating a subject with cancer, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for inhibiting proliferation of the cells of the cancer.

5.7. AUTOIMMUNE DISEASES

Dietary zinc deficiency is linked to autoimmune diseases in mice (R. S. Beach *et al.*, 1982, J. Immunol., 129, 2686). In one embodiment of the present invention, oxidizing compounds are administered to subjects at risk for or experiencing autoimmune diseases so as to promote the release of zinc from MT and thus ameliorate the disease. Thus in one embodiment, the invention provides a method of treating a subject with an

autoimmune disease comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the autoimmune disease.

5.7.1. SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus has been associated with dietary zinc deficiency (*id.*). In one embodiment of the present invention, oxidizing compounds are administered to subjects at risk for or experiencing systemic lupus erythematosus so as to promote the release of zinc from MT and thus ameliorate the disease. Thus, in one embodiment, the invention provides a method of treating a subject with systemic lupus erythematosus comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the lupus erythematosus.

5.8. VIRAL DISEASES

5.8.1. AIDS AND HIV INFECTION

Zinc plays an important role in several of the steps in the life-cycle of HIV-1.

For example, integration of HIV-1 DNA into host-cell genome is an essential step in the HIV-1 life cycle, thus interruption of this integration is a means of combatting HIV-1 infection. HIV-1 integration requires cleavage of two nucleotides from the 3' ends of viral DNA by HIV-1 integrase. HIV-1 integrase contains three distinct domains (J. Kukosky and A. M. Skalka, 1994, Pharmacol. Ther., 61, 185), one of which (the N-terminal domain) is known to bind zinc (M. S. Johnson *et al.*, 1986, Proc. Natl. Acad. Sci. USA, 83, 7468). Zinc binding has been shown to play important structural and functional roles in integrase activity (S.P. Lee *et al.*, 1997, Biochemistry, 36, 173).

Additionally, mature HIV-1 nucleocapsid protein (NCP0 requires a zinc atom to reach its folded conformation and removal of zinc from NCP prevents the operation on it by viral protease (E. W. Wondrak *et al.*, 1994, J. Biol. Chem., 269, 21948).

Furthermore, dietary intake of zinc has been associated with poorer survival of AIDS patients (A. M. Tang *et al.*, 1996, Am. J. Epidem., 143, 1244).

In one embodiment of the present invention, reducing compounds are administered to a patient infected with HIV so as to prevent zinc transfer from MT to HIV-1 integrase and thus to inhibit HIV-1 integrase activity and also so as to prevent transfer of zinc from NCP to T, so as to interrupt the HIV-1 life cycle. Thus in one embodiment, the invention provides a method of treating a subject suffering from an infection with HIV

comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the infection with HIV.

5.8.2. ILARVIRAL AND A1MV INFECTIONS

Chelation of zinc has been demonstrated to reduce RNA-dependent RNA polymerase in alfalfa mosaic virus (R. Quandt and E. M. J. Jaspars, 1991, FEBS, 278, 61). Such inhibition has been linked to a particular capsid protein containing a zinc-finger region (P. C. Sehnke and J. E. Johnson, 1994, Virology, 204, 843). Zinc is thus believed to be important for infectivity of A1MV and ilarviruses (*id.*).

In one embodiment of the present invention, reducing compounds are administered to plants at risk for or undergoing ilarviral and related viral infection such as A1MV so as to prevent transfer of zinc from MT to the viral capsid protein and thereby reduce or prevent infection. Thus in one embodiment, the invention provides a method of treating a plant or plant cell infected with an ilarvirus or a mosaic virus comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein amount effective for treatment of the infection with the ilarvirus or the mosaic virus.

5.8.3. PAPILLOMA VIRAL CARCINOGENESIS

E7 proteins of cancer-involved HPVs are cysteine-rich zinc-binding proteins. (E. J. Roth *et al.*, 1992, J. Biol. Chem., 267, 16390). In one embodiment of the present invention, reducing compounds of the present invention are administered to a patient infected with or in danger of infection with cancer-involved HPVs so as to prevent transfer of zinc from MT to the HPV E7 protein and thereby to prevent or mitigate cervical cancer. Thus, in one embodiment, the invention provides a method of treating a subject infected with papilloma virus, said method comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the papilloma virus.

5.8.4. SEMLIKI FOREST VIRUS

Zn^{2+} has been shown to *in vitro* to inhibit SFV-liposome fusion due to interference with E1 protein trimer formation (J. Corver *et al.*, 1997, Virology, 238, 14). In one embodiment of the present invention, an animal in need of treatment for SFV infection is treated with an oxidizing therapeutic so as to induce zinc transfer from metallothionein to the SFV E1 protein and thereby to inhibit access of SFV to the host cytosol. Thus, in one

embodiment, the invention provides a method of treating a subject infected with Semliki Forest virus comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the Semliki Forest virus.

5.8.5. HEPATITIS C

Several Hepatitis C (HCV) non-structural proteinases (NSP) exhibit zinc-dependent activity (A. Grakoui *et al.*, 1993, J. Virol., 67, 2832; M. Hijikata *et al.*, 1993, J. Virol., 67, 4665). Activity of these proteinases is necessary for the replication of the HCV genome. At least one of the NSP, NS3 has a zinc-binding site (R. A. Love *et al.*, 1996, Cell, 87, 332).

In one embodiment of the present invention, reducing compounds are administered to a patient infected with HCV so as to prevent MT transfer of zinc to HCV NS3, and thereby to inhibit further replication of the HCV genome. Thus, in one embodiment, the invention provides a method of treating a subject infected with hepatitis C virus comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the hepatitis C virus.

5.8.6. MEASLES

One result of measles virus (MV) infection is production of MV V protein and diffuse cytoplasmic distribution (E. A. Wardrop and D. J. Briedis, 1991, J. Virol., 65, 3421). MV V protein is believed to play a role in transcription and/or replication of MV genome. V protein binds zinc (P. Liston and D. J. Briedis, 1994, Virol., 198, 399).

In one embodiment of the present invention, a reducing compound is administered to a patient who has been exposed to measles so as to prevent the transfer of zinc from MT to MV V protein. Thus, in one embodiment, the invention provides a method of treating a subject infected with measles virus comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the measles virus.

5.9. COFACTOR ADDITION

In any of the embodiments above, cofactors may be co-administered concurrently with or within a time period just prior to administration of the compound in order to provide target specificity to the zinc released. Preferably, the cofactors are administered from 0.01 seconds to 24 hours prior to administration of the compound.

Cofactors useful for co-administration with the compounds of the present invention include but are not limited to ATP, GTP and GSH.

5.10. SITE SPECIFIC TARGETING

Single-chain fragment MT containing anti-carcinoembryonic antigen antibody has been proposed as a diagnostic imaging agent in colorectal cancer (G. A. Pietersz *et al.*, 1998, J. Nucl. Med., 39, 47). In one embodiment of the present invention, an MT derivative bound to antibody to a cellular antigen is coadministered with an oxidant or reductant, to target particular sites for release or inhibition of release of zinc.

5.11. THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a compound described above. The subject is preferably an animal, including but not limited to animals such as cows, pigs, chickens, primates, etc., and is preferably a mammal, and most preferably human.

Various delivery systems are known and can be used to administer a compound according to the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct

injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

Table 2. Preferred forms of administration of compounds as a function of condition

Condition	Preferred Forms of Administration
Disorders of the central nervous system	Intravenous
Inflammatory diseases	Topical; Oral; Inhalation; Intravenous
Disorders of the endocrine system	Oral; Intravenous
Apoptosis and injury from radiation or chemotherapy	Intravenous; Topical
Carcinogenesis	Intravenous; oral
Autoimmune diseases	Intravenous; oral

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile. The formulation should suit the mode of administration.

The composition, if desired, can also contain wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed

with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The therapeutic compounds according to the invention may also be immobilized, for example, on a biologically-inert non-polymeric or polymeric support. Non-limiting examples of such supports include functionalized polystyrene or other polymeric beads, fibers, sheets, etc, and functionalized cellulosic materials such as paper, cotton thread, and the like. Immobilization may involve covalent attachment to the support, or inclusion, adsorption or absorption on or into a porous, non-porous or swellable support. Such immobilization permits easy introduction of the compounds to a specific site by administering the compound on the support, and also provides for easy later removal by removal of the support.

The compounds of the invention may also be formulated for transdermal and transmucosal administration. One of ordinary skill would understand that there are numerous technologies available for carrying out such transdermal and transmucosal administration.

The amount of the compound of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

6. EXAMPLES

6.1. THE GLUTATHIONE REDOX COUPLE MODULATES ZINC TRANSFER FROM METALLOTHIONEIN TO ZINC-DEPLETED SORBITOL DEHYDROGENASE

The release and transfer of zinc from metallothionein (MT) to zinc-depleted sorbitol dehydrogenase *in vitro* has been used to explore the role of MT in cellular zinc distribution. A 1:1 molar ratio of MT to sorbitol dehydrogenase is required for full reactivation, indicating that only one of the seven zinc atoms of MT is transferred in this process. Reduced glutathione (GSH) and glutathione disulfide (GSSG) are critical modulators of both the rate of zinc transfer and the ultimate number of zinc atoms transferred. GSSG increases the rate of zinc transfer 3-fold and its concentration is the major determinant for efficient zinc transfer. GSH has a dual function: In the absence of GSSG, it inhibits zinc transfer from MT, indicating that MT is in a latent state under the relatively high cellular concentrations of GSH. In addition, it primes MT for the reaction with GSSG by enhancing the rate of zinc transfer 10-fold and by increasing the number of zinc atoms transferred to four. ^{65}Zn-labeling experiments confirm the release of one zinc from MT in the absence of glutathione and the more effective release of zinc in the presence of GSH and GSSG. *In vivo*, MT may keep the cellular concentrations of free zinc very low and, acting as a temporary cellular reservoir, release zinc in a process that is dynamically controlled by its interactions with both GSH and GSSG. These results suggest that a change of the redox state of the cell could serve as a driving force and signal for zinc distribution from MT.

Thus, in MT *the protein* plays a role in the biological function *of zinc*, a paradigm quite different from that in most other zinc proteins where *zinc* plays a role in the biological function *of the protein*. The compounds of the present invention take advantage of the tight binding of zinc to MT and the ability to control its release. In one embodiment, the present invention is directed to glutathione disulfide mediated zinc release in the

presence of reduced glutathione. We have now discovered that such zinc transfer reactions are regulated dynamically by interactions between MT and the reduced glutathione (GSH)/GSSG couple. We exploit the fact that zinc distribution is not dependent on MT alone, but rather on a biochemical system in which MT and glutathione interact cooperatively. Such a glutathione/MT system allows MT to serve as both a cellular reservoir for zinc and a controlled release system that can supply different amounts of zinc according to therapeutic requirements. Biological specificity of this system is not embedded in the recognition between MT and zinc acceptor molecules, but rather in signals effecting a change of the cellular redox state and in signals that control the availability of the zinc acceptor molecules.

MATERIALS AND METHODS

Materials. GSH, GSSG, NAD^+, pyridine-2,6-dicarboxylic acid (dipicolinic acid), Coomassie Brilliant Blue G, 2-carboxyl-2'-hydroxy-5'-sulfoformazylbenzene (Zincon) and carbonic anhydrase (bovine erythrocytes) were from Sigma; sorbitol and 4-(2-pyridylazo)resorcinol (PAR) from Aldrich; $^{65}ZnCl_2$ (77.7-103.6 Gbq/g) from Dupont/NEN. **Preparation and Characterization of Human MT.** Human MT-1 and MT-2 isoforms were prepared in this laboratory according to established procedures known to those of ordinary skill (see, for example, M. Vašák, 1991, Methods Enzymol. 205, 41-44) and converted to their apoforms and reconstituted with Zn^{2+} to result in the respective Zn_7-MT isoforms (see, W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241). Excess zinc was removed by gel filtration through a Sephadex G-50 fine column (30 x 0.5 cm) and the MT isoforms were characterized by metal analyses, determinations of sulfhydryl groups with dithiodipyridine, and amino acid analyses. Loosely bound zinc in MT solutions was assayed spectrophotometrically at 620 nm with Zincon. Addition of 10 µl of 10 mM Zincon to 890 µl of 10 mM Tris-HCl, pH 8.6 identifies the existence of less than 4% of loosely bound zinc.

Preparation and Characterization of SDH and Zinc-depleted SDH. Sheep liver SDH was obtained as a lyophilized powder from Boehringer Mannheim and stock solutions were prepared by dissolving enzyme in 1 ml of 0.2 M Tris-HCl, pH 7.4. As is standard in the art, enzyme concentrations were determined with a Coomassie Blue protein-dye binding assay with bovine serum albumin as a standard (see, R. K. Scopes, 1982, Protein Purification (Springer, New York), p 266).

Zinc-depleted SDH (apo-SDH) was prepared according to standard procedures familiar to those of ordinary skill, in Centricon-10 centrifugal microconcentrators (Amicon) using 0.2 M phosphate buffer, pH 7.0, containing 10 mM dipicolinic acid to remove zinc (see, for example, W. Maret, 1989, Biochemistry, 28, 9944-9949). The apoform had 2.0% residual enzymatic activity compared with the native zinc enzyme and contained 0.015 g-atom zinc/subunit. The amount of zinc in native SDH was 1 g-atom/subunit, in agreement with the expected value (J. Jeffery *et al.*, 1984, EMBO J. 3, 357-360).

Kinetic Studies of the Reconstitution of Zinc-depleted SDH. Different amounts of free zinc, MT or carbonic anhydrase were incubated with 1.7 μM apo-SDH in 0.2 M Tris-HCl buffer, pH 7.4 at 22.5 ± 0.5 °C. Aliquots (10 μl) were withdrawn periodically and assayed for enzymatic activity. The assay solution consisted of 50 mM sorbitol and 1.5 mM NAD^+ in a total volume of 1 ml of 0.2 M Tris-HCl buffer, pH 7.4. Enzyme activity was determined spectrophotometrically (U. Gerlach, (1965) in Methods of Enzymatic Analysis, ed. Bergmeyer, H. U. (Academic Press, New York), pp. 761-764) by measuring the rate of absorbance change accompanying NAD^+ reduction at 25.0 ± 0.5 °C. The experimental error between assays did not exceed 5%.

Reconstitution of Zinc-depleted SDH with $^{65}Zn_7$-MT-2. Aliquots of reactants (molar ratio of zinc to apo-SDH of 1.0) were incubated at 22.5 ± 0.5 °C for 60 min. Reaction mixtures were then separated on a DEAE MemSep-1000 chromatography cartridge (Millipore) using a linear, 10-min gradient from 0 to 75 mM NaCl in 10 mM Tris-HCl, pH 8.6 at a flow rate of 5 ml/min. Radioactivity in each fraction was measured by γ-emission spectroscopy with a Searle model 1185 Automatic Gamma System operating at a 0.12-1.2 MeV energy range.

Kinetic Studies of the Reaction between MT-2 and PAR. PAR (100 μM) was incubated with MT (1.3 μM) and the reaction was followed by measuring the increase of absorbance at 500 nm of the $Zn(PAR)_2$ complex (J. B. Hunt *et al.*, 1985, Anal. Biochem., 146, 150-157; C. F. Shaw III *et al.*, 1990, Inorg. Chem., 29, 403-408).

RESULTS

Kinetic Studies of the Reconstitution of Zinc-depleted SDH with Zinc Ions and MT. Mammalian SDH was used an exemplary cellular zinc acceptor. Mammalian SDH is a tetramer of four identical subunits with an overall molecular mass of 152 kDa and one catalytic zinc atom per subunit (J. Jeffery, *et al.*, 1984, EMBO J., 3, 357-360; J. Jeffery, *et*

al., 1981, Eur. J. Biochem., 120, 229-234; W. Maret, & D. S. Auld, 1988, Biochemistry, 27, 1622-1628). The reconstitution of apo-SDH with free zinc ions is very fast and reaches 100% within 3 min (Figure 1, upper curve). Standard kinetic treatment of the slopes of plots of [Zn-SDH]/[Zn] vs. time gives an estimated rate constant for reconstitution of apo-SDH with free zinc of 20,000 $M^{-1}s^{-1}$ (Table 3).

Table 3. Rate of zinc transfer to apo-SDH

Zinc donor	Rate constants, $M^{-1}s^{-1}$
free zinc	20000
MT-1	24
MT-2	16
MT-1+GSH	20
MT-1+GSSG	79

Rate constants were obtained from plots for a second-order reaction:

$$[SDH]/[Zn] = k_2[\text{apo-SDH}]_0 t.$$

Reconstitution of SDH with Zn-MT-1 is much slower (Figure 1, lower curve) than with free zinc and reaches 17% after one hour at equimolar concentrations of apo-SDH and zinc in MT. In addition, a small burst of reactivation occurs with MT-1 at the first time point of the measurements, perhaps due to the traces of zinc in MT that can be dechelated by treatment with Zincon (see, T. Li *et al.*, 1980, Proc. Natl. Acad. Sci. USA, 77, 6334-6338). MT-2 reactivates apo-SDH to 13% (data not shown) and is therefore slightly less effective in transferring zinc than MT-1. The second-order rate constants for MT-1 and MT-2 are 24 and 16 $M^{-1}s^{-1}$, respectively (Table 3). These slightly different rates and degrees of reactivation with MT-1 and -2 are in accord with data reported for carbonic anhydrase and rat liver MT isoforms (D. R. Winge and K. A. (Miklossy, 1982, Arch. Biochem. Biophys., 214, 80-88).

Zinc Transfer from Carbonic Anhydrase to Zinc-depleted SDH. To determine whether or not zinc transfer between MT and apo-SDH is merely controlled by the thermodynamic equilibrium between MT and the apoenzyme, carbonic anhydrase was investigated for its potential to transfer zinc to apo-SDH. Bovine carbonic anhydrase binds zinc less tightly

[$K_D = 1 \times 10^{-12}$ M at pH 7.0 (S. Lindskog and B. G. Malmström, 1962, J. Biol. Chem., 237, 1129-1136)] than human MT [$K_D = 1.4 \times 10^{-13}$ M at pH 7.0 (J. H. R. Kägi, (1993) in Metallothionein III, eds. Suzuki, K. T., Imura, N. & Kimura, M. (Birkhäuser, Basel), pp. 29-55)] and, therefore, should be a better zinc donor to apo-SDH if zinc transfer is simply controlled by the difference in the zinc binding constants of the apoenzyme and MT. After a one hour incubation of apo-SDH and carbonic anhydrase no significant zinc transfer is observed when there is a seven-fold molar excess of carbonic anhydrase in terms of zinc (Fig.2, lower curve). In contrast, zinc transfer with MT is very efficient (Figure 2, upper curve). Therefore, zinc transfer from MT must be under kinetic control. High kinetic lability of zinc distinguishes MT from other zinc proteins, so that MT can serve as an efficient source of zinc.

Reconstitution with Different Ratios of MT and Zinc-depleted SDH. SDH activity increases linearly as a function of the ratio of free zinc (Figure 3A) or of MT-1 (Figure 3B) to apo-SDH until its zinc content is fully restored. The equivalence point in the titration of apo-SDH with free zinc ions is at 1 g-atom of zinc per subunit (Figure 3A). In contrast, one molecule of MT, which contains seven zinc atoms, is needed per subunit of apo-SDH to achieve full reactivation (Figure 3B). Thus, under these conditions only one of the seven zinc atoms is transferred from MT, which explains why only 17% of the enzymatic activity is restored when equimolar concentrations of enzyme and zinc are employed (Figure 1).

Reconstitution of Zinc-depleted SDH with MT in the Presence of GSSG and/or GSH. GSSG is known to mobilize all seven zinc atoms from MT at pH 8.6 (W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241). We therefore tested how GSSG effects the transfer of zinc atoms at pH 7.4. Adding MT to apo-SDH in the presence of GSSG generates activity faster and to a greater extent than in its absence. Thus, incubation of apo-SDH with MT-1 (equimolar zinc) for one hour in the presence of GSSG leads to 31% reactivation, almost twice that achieved without GSSG (Figure 4). Under these conditions the second-order rate constant increases 3-fold to 79 $M^{-1}s^{-1}$ (Table 3), a comparison based on the assumption that only one zinc atom is released. In fact, however, GSSG releases more than one metal atom. Incubating MT with GSSG for five hours prior to the addition of apo-SDH results in a large burst of reactivation up to 44% (data not shown). The reaction does not approach the rate at which free zinc ions interact with apo-SDH, indicating that a step other than the reconstitution of apo-SDH with free zinc becomes rate-limiting. Most likely, this step is the release of zinc from MT.

GSH has been shown to bind to rabbit liver Cd_5Zn_2-MT (M. Brouwer *et al.*, 1993, Biochem. J., 294, 219-225). We therefore examined the reconstitution of apo-SDH with MT-1 in the presence of GSH. Importantly, a slight inhibition of GSH on zinc transfer (Figure 4) was observed in three independent experiments.

Zinc transfer from MT to apo-SDH was then investigated in the presence of both GSH and GSSG. Neither GSH nor GSSG at the concentrations employed affects the activity of SDH in the absence of MT. Nevertheless, GSH strongly influences the capacity of MT-2 to reconstitute apo-SDH in the presence of GSSG (Table 4). Thus, it further modulates the enhancement of zinc transfer from MT to apo-SDH by GSSG (Figure 4). At a constant concentration of 3.0 mM GSSG, the rate and extent of zinc transfer from MT-2 to apo-SDH increase until a plateau is reached at the GSH concentration of 1.5 mM (Figure 5A & Table 4). Under these conditions, the rate constant is increased 10-fold and the amount of zinc transferred is 5-fold greater than that in the absence of GSH and GSSG. When the concentration of GSH was kept constant at 1.5 mM and the concentration of GSSG was varied, the rate and extent of zinc transfer increased linearly with increasing concentrations of GSSG (Figure 5B & Table 4).

Table 4. Modulation of the rate of zinc transfer from MT-2 to apo-SDH by GSH and GSSG

[GSH], mM	[GSSG], mM	[GSH]/[GSSG]	Rate constants*, $M^{-1}s^{-1}$
0	3	0	81.
0.375	3	0.125	101.
0.75	3	0.25	120.
1.2	3	0.4	183.
1.5	3	0.5	204.
3.0	3	1.0	187.
6.0	3	2.0	203.
1.5	0	/	30.[1]
1.5	0.375	4.0	44.
1.5	1.5	1.0	124.
1.5	3.0	0.5	204.

*Rate constants were calculated as described in Table 3.
[1]The apparent slight activation noted here in comparison with the data in Table 3 is likely due to small amounts of GSSG formed in the 1.5 mM GSH solution.

The measurement of zinc transfer by means of the recovery of SDH activity determines the amount of zinc incorporated into the active site of SDH, but only inferentially that of zinc released from MT. Therefore, ^{65}Zn-MT was employed to determine zinc release and transfer to apo-SDH directly. $^{65}Zn_7$-MT-2 was incubated with apo-SDH for one hour (molar ratio of zinc to apo-SDH of 1.0) in the absence of GSH and GSSG, followed by separation of the mixture by anion-exchange chromatography and analysis. Radioactivity in the MT-2 fractions (fractions 14-17) decreases while it increases in the fractions corresponding to SDH (fractions 1-5) (Figure 6). Quantification proves that only one (1.1 ± 0.1, n=3) of the seven zinc ions is released from MT, confirming the stoichiometry obtained based on the recovery of SDH activity (Figure 3B). In the presence of GSH/GSSG, more than one zinc is released from MT-2 and transferred to apo-SDH (Figure 6B). At a GSH/GSSG ratio of 0.5, four of the seven zinc ions are transferred from MT-2 to apo-SDH. Concomitantly, a new radioactive species is formed (fractions 9-11) (Figure 6B), whose molecular identity is at present unknown. These ^{65}Zn-labeling

experiments provide direct evidence that the GSH/GSSG redox couple modulates zinc transfer from MT to another protein and that the extent of zinc transferred depends on the redox ratio.

Zinc Transfer from MT to PAR in the Presence of Glutathione. In order to determine whether or not GSH affects zinc transfer in another system, the reaction of MT-2 with PAR, a chromophoric dye with a zinc binding constant of $10^{12.6}$ M^{-1} at pH 7.4 (C. F. Shaw III *et al.*, 1991, Methods Enzymol., 205, 401-414) was used. GSH inhibits transfer of one zinc atom in a concentration-dependent manner, yielding 75% inhibition at a concentration of 3 mM GSH (Figure 7). We attempted to fit the data to a simple model (dotted line) with one GSH binding site and a dissociation constant of 56 μM, which is slightly higher than the value of 15 μM reported for rabbit Cd_5Zn_2-MT (M. Brouwer *et al.*, 1993, Biochem. J. 294, 219-225). This simple binding model is clearly unsatisfactory due to the complex mechanism of interactions between GSH and MT.

DISCUSSION

Zinc Transfer in the Absence of Glutathione. *In vitro*, MT transfers zinc to zinc-depleted SDH and fully restores enzymatic activity. The stoichiometry of the interaction of apo-SDH with MT is most striking (Figs. 3 and 6). One MT molecule is needed to supply each subunit of SDH with one zinc, although each MT molecule contains seven zincs. Thus only one of the zinc atoms of MT appears to be available for transfer to apo-SDH.

It has been observed that in Cd_5Zn_2-MT the two zinc atoms reside at defined positions in the β-domain (A. H. Robbins *et al.*, 1991, J. Mol. Biol., 221, 1269-1293) and that metals in this domain exchange more rapidly than those in the α-domain (W. Maret *et al.*, 1997, Proc. Natl. Acad. Sci. USA, 94, 2233-2237; D. G. Nettesheim *et al.*, 1985, Biochemistry, 24, 6744-6751). These finding suggest that the zinc atom transferred from MT-2 to apo-SDH is from the β-domain. An NMR study on Cd_6-MT, from which one cadmium atom had been removed from the β-domain with EDTA (F. Vazquez and M. Vašák, 1988, Biochem. J., 253, 611-614), provides further insight into the structure of MT after one zinc atom has been transferred. In this case, removal of one cadmium atom allows redistribution of the remaining cadmiums within the β-domain and yields equal but less than stoichiometric occupancies of all these metal binding sites in this domain. A similar situation has been observed for the 3-metal site of dehydroascorbate oxidase. Crystal structure analyses of the enzyme in which "Type 2" copper had been removed revealed that the remaining two copper ions scramble among the three available binding sites (A. Messerschmidt *et al.*, 1992, Eur. J. Biochem., 209, 597-602).

Studies with more cellular relevance have led to a concept which postulates that a concerted action between MT, GSH, and GSSG is required to effect zinc transfer and that MT *per se* cannot be a transfer agent *in vivo* in the specific sense of the word. Although we found that the binary system MT/apoenzyme functions *in vitro*, it may not apply *in vivo* since MT interacts with GSH and zinc transfer from the MT/GSH complex is inhibited.

Zinc Transfer in the Presence of Glutathione. GSSG, which releases zinc from MT, accelerates the rate and dramatically increases the extent of reactivation of apo-SDH by MT (Figure 5). Moreover, GSH stimulates the rate of zinc transfer in the apo-SDH/MT/GSSG system, signifying control of the MT molecule by glutathione with important implications regarding modulation of zinc transfer by the GSH/GSSG redox couple *in vivo* (see below).

In terms of molecular mechanism, GSH and GSSG could i) bind to SDH and affect its activity, ii) control the amount of free zinc available once it is released from MT, or iii) bind to MT and affect its conformation and zinc binding. Our data strongly favor the last mechanism. Molecular modeling studies suggest that GSH binds in a cleft of the β-domain and its thiol sulfur displaces the thiol ligand of Cys-26 of the zinc atom designated as Zn-2 in the crystal structure of MT (M. Brouwer *et al.*, 1993, Biochem. J. 294, 219-225). The exposed MT thiol sulfur could then undergo thiol/disulfide interchange with GSSG, explaining why GSH stimulates the rate of the reaction between MT and GSSG. GSH binding would protect MT from the loss of Zn-2, thereby inhibiting its transfer, while providing a reactive thiol for the reaction with GSSG and resulting in zinc transfer through a process that is strictly proportional to and dependent on the concentration of GSSG (Figure 5B). This zinc distribution system seems to rely on specific interactions between MT and glutathione and to respond to signals that change the cellular GSH/GSSG redox state (see below) rather than on the mutual molecular recognition of MT and the apoprotein. Hence, a system consisting of MT/GSH/GSSG provides a mechanism to control zinc transfer among a large variety of acceptors. Thus regulation at the protein level can now be added to the already known extensive and intricate regulation of MT gene expression (G. K. Andrews, 1990, Prog. Food Nutr. Sci., 14, 193-258). Both types of regulation support key functions of MT in cellular zinc traffic.

MT Controls Cellular Free Zinc. The reactivation of apo-SDH with Zn-MT occurs at a rate that is slower than that with free zinc ions (Figure 1). Specifically, the rate constant for zinc transfer from MT to apo-SDH is three orders of magnitude less than that of about 20,000 $M^{-1}s^{-1}$ estimated for apo-SDH and free zinc ions. In the few other enzyme systems studied (A. O. Udom and F. O. Brady, 1980, Biochem. J., 187, 329-335), MT also failed to accelerate the reconstitution of the apoenzyme, as might have been expected if MT had

insertase-like enzymatic activity such as, e.g. ferrochelatase, the enzyme that inserts iron into protoporphyrin IX. At best, the rate of zinc transfer is as rapid as that of free zinc ions in the case of apo-carbonic anhydrase (T. Y. Li *et al.*, 1980, Proc. Natl. Acad. Sci. USA, 77, 6334-63387). At first glance, such a lack of any kinetic advantage might argue against a physiological function of MT as a universal zinc donor for the apoforms of zinc proteins. However, a comparison between free zinc ions and MT as zinc donors is not appropriate, because there is very little free zinc in the cell to serve the purpose. Intracellular zinc concentrations are exceedingly low, i.e. <100 pM (E. J. Peck and W. J. Ray, 1971, J. Biol. Chem., 246, 1160-1167; T. J. B. Simons, 1991, J. Membr. Biol., 3, 63-7; D. Atar *et al.*, 1995, J. Biol. Chem., 270, 2473-2477). One of the roles of MT, therefore, seems to be to ensure that zinc concentrations are maintained at such low levels. MT can achieve this by binding zinc very tightly [$K_D = 1.4 \times 10^{-13}$ M for human Zn-MT at pH 7]. If metals were supplied to apoproteins in the form of free ions, zinc would compete with other metal ions for the binding site. This is apparently not the case, since purified zinc enzymes, at least those from animal sources, invariably contain only zinc despite the fact that other metal ions can bind to the same site *in vitro*, sometimes even with partial or full conservation of catalytic or other function. Thus, the choice of a particular metal ion must be "directed by its cellular availability and mobilization processes rather than by its chemical nature" (Z. Dauter *et al.*, 1996, Proc. Natl. Acad. Sci. USA, 93, 8836-8840) or by the coordination environment provided by the protein. It would seem more likely that specificity of metal incorporation is controlled by proteins and that protein-bound zinc is the source of zinc for apoproteins. MT can serve such a function and can make zinc available in a controlled manner. As the above experiments show, zinc is available from MT despite its relatively high thermodynamic stability, and this property distinguishes it from other zinc proteins where zinc does not exchange on a similar time scale (W. Maret *et al.*, 1997, Proc. Natl. Acad. Sci. USA, 94, 2233-2237). The kinetic lability of zinc in MT becomes strikingly apparent when compared to carbonic anhydrase, in which zinc has a similar thermodynamic stability, but is kinetically much less capable of zinc transfer (Figure 2).

Implications for Zinc Transfer *in vivo*. MT maintains a low cellular concentration of free zinc (in the picomolar range) and yet, as our data suggest, provides zinc to appropriate acceptors in reactions that are modulated by GSH/GSSG. Most interestingly, the rate and amount of zinc released from MT depend on the relative concentrations of both GSH and GSSG, suggesting that zinc distribution from MT is controlled dynamically by the cellular GSH/GSSG state. What do these *in vitro* studies imply about zinc transfer *in vivo*? GSH alone inhibits zinc transfer (Figures 4 and 7). Thus, under the prevailing, reducing

conditions in the presence of almost millimolar concentrations of GSH, MT may not transfer zinc to apo-proteins in the normal cellular environment where the GSH/GSSG redox ratio is between 30:1 to 100:1 (C. Hwang *et al.*, 1992, Science 257, 1496-1502). It is noteworthy that these are steady-state conditions under which there may not be any need for zinc in the cell, and where the role of MT is to sequester zinc, not to distribute it. Hence, this condition must be perturbed in order to change the role of MT from that of an acceptor to that of a donor when zinc is needed in events such as cell proliferation, for example. We show here that this can be achieved by lowering the GSH concentration or by changing the GSH/GSSG redox state. The cellular concentration of GSH can vary over almost two orders of magnitude (A. Meister, 1988, J. Biol. Chem., 263, 17205-17208). Therefore, transfer of but one zinc atom might have physiological significance at low concentrations of GSH and under a regime of demand for zinc which is quite different from that at high concentrations of GSH. It was shown earlier that high concentrations of GSSG release zinc from MT , but the effect of GSH was not examined. We demonstrate here that the presence of GSH actually enhances the effect of GSSG. A role of both GSH and GSSG in the process of zinc release creates conditions *in vitro* that approach those *in vivo*. The rate of zinc transfer depends linearly on the amount of GSSG (Figure 5), i.e. the more oxidative the redox state becomes, the more efficiently is zinc transferred to a suitable acceptor (Figures 4-6). These observations seem to link the cellular GSH/GSSG redox state and its control circuitry to the biochemistry of MT.

At present we do not yet know the precise range in which the cellular GSH/GSSG redox state changes, the magnitude of changes that lead to zinc distribution *in vivo*, where the process takes place, and whether only some or all apo-proteins receive zinc from MT. However, it is clear that zinc transfer from MT to other proteins actually occurs *in vivo* (M. G. Cherian, 1977, J. Nutr., 107, 965-972). It is noteworthy that substantial deviations from the cytoplasmic GSH/GSSG redox ratio (30:1 to 100:1) control events crucial in signal transduction and gene transcription (H. P. Monteiro and A. Stern, 1996, Free Radic. Biol. Med., 21, 323-333; C. K. Sen and L. Packer, 1996, FASEB J., 10, 709-720; Y. Sun and L. W. Oberley, 1996, Free Radic. Biol. Med., 21, 335-348). This may be achieved by enzymatically generating high local concentrations of GSSG, or by compartmentalization of the process. Thus, in some compartments of the cell such as the endoplasmic reticulum the GSH/GSSG redox ratio is between 3:1 and 1:1 (C. Hwang *et al.*, 1992, Science, 257, 1496-1502), conditions that are quite similar to those under which we find efficient zinc transfer *in vitro*. The modulation of the reaction between MT and GSSG by the amount of GSH (Figure 5A) also occurs in the range where the concentration of GSH changes in the cell.

For example, cellular GSH concentrations are relatively high and vary in the range 0.1-10 mM (A. Meister, 1988, J. Biol. Chem., 263, 17205-17208). Finally, even if the concentrations of GSSG in our experiments appear to be relatively high compared to what they might be maximally in the cell, they certainly need not be as high as 3 mM to exert an appreciable effect (Figure 5B). It is also important to note that we have observed higher efficiency in zinc release with disulfides other than GSSG (W. Maret, 1995, Neurochem. Int., 27, 111-117). Thus, in the cell, a more reactive disulfide or other compounds might assume the *in vitro* role of GSSG.

6.2. CONTROL OF ZINC TRANSFER BETWEEN THIONEIN, METALLOTHIONEIN AND ZINC PROTEINS

Metallothionein (MT), despite its high metal binding constant ($K_{Zn} = 3.2 \times 10^{13}\ M^{-1}$ at pH 7.4), can transfer zinc to the apo-forms of zinc enzymes that have inherently lower stability constants. To gain insight into this apparent paradox, zinc transfer between zinc enzymes and MT has been studied. Zinc can be transferred in both directions, i.e. from the enzymes to thionein, the apo-form of MT, and from MT to the apo-enzymes. Agents that mediate or enhance zinc transfer have been identified which provide kinetic pathways in either direction. MT does not transfer all of its seven zinc atoms to an apo-enzyme, but apparently contains at least one that is more prone to transfer than the others. Modification of thiol-ligands in MT zinc clusters increases the total number of zinc ions released and, hence the extent of transfer. Aside from disulfide reagents we show that selenium compounds are cellular enhancers of zinc transfer from MT to apo-enzymes. Zinc transfer from zinc enzymes to thionein, on the other hand, is mediated by zinc chelating agents such as Tris buffer, citrate or glutathione. Redox-agents are asymmetrically involved in both directions of zinc transfer. For example, reduced glutathione mediates zinc transfer from enzymes to thionein, while glutathione disulfide oxidizes MT with enhanced release of zinc and transfer of zinc to apo-enzymes. Therefore, the cellular redox state and the concentration of other biological chelating agents might well determine the direction of zinc transfer and ultimately affect zinc distribution.

Metallothionein (MT) is a protein that has long been in search of a function. Its composition and structure (Margoshes, M. and B. L. Vallee, 1957, J. Am. Chem. Soc., 79, 4813; A.H. Robbins *et al.*, 1991, J. Mol. Biol., 221, 1269-1293; K. Wüthrich, 1991, Methods Enzymol., 205, 502-520) have clearly identified it as a biological metal chelating agent yet it does not resemble conventional proteins and its two distinct zinc-sulfur clusters are unlike any known inorganic zinc(II) complexes. Two properties of MT reveal aspects of

its cellular function(s). Its binding of zinc is exceptionally strong owing to the exclusive coordination of the metal with cysteine sulfur ligands (stability constant of Zn_7MT-2 = 3.2 x $10^{13} M^{-1}$ at pH 7.4 (J. D. Otvos *et al.*, 1989, Comments Inorg. Chem., 1, 1-35). Thionein (T), the apo-form, is a potent zinc acceptor. On the other hand, the sulfur ligands are highly reactive and determine not only the binding of zinc to T but also its release from MT (H. Fliss, and M. Ménard, 1992, Arch. Biochem. Biophys., 293, 195-199; W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241), which then becomes a zinc donor. This raises questions as to the circumstances under which T removes zinc from proteins and/or MT donates it to apo-proteins. Zinc would be expected to be transferred from the protein with the lower stability constant to that with the higher one (reaction 1) which in most cases would determine unidirectional zinc flow from the zinc protein to T.

$$\text{Zn-Donor} + \text{T} \rightleftharpoons \text{ZN-MT} \rightleftharpoons \text{T} + \text{Zn-Acceptor}$$

(reaction 1)

Indeed, T has been shown to block the action of zinc-dependent transcription factors, e.g. TF IIIA and Sp1, presumably via metal chelation (J. Zeng *et al.*, 1991, FEBS Lett., 279, 310-312; J. Zeng *et al.*, 1991, Proc. Natl. Acad. Sci. USA, 88, 9984-9988). MT, on the other hand, transfers at least some of its complexed zinc ions to a number of apo-proteins (A. O. Udom and F. O. Brady, 1980, Biochem. J., 187, 329-335). The process by which this is accomplished remains enigmatic, since the zinc binding constants of most of the enzymes studied are at least 1,000 times lower than that of MT. What conditions and factors allow T to remove zinc from proteins and for MT to donate zinc to apo-proteins? The dual capacity of this system to function as both a zinc donor and acceptor (reaction 1) clearly calls for critical examination.

To our knowledge, attempts have not been made to study bidirectional zinc transfer between a specific protein and T/MT in order to examine the acceptor/donor properties of the T/MT system. We have now employed two different zinc enzymes for this purpose, *E. coli* alkaline phosphatase (AP) and bovine carboxypeptidase A (CPA). They were chosen because their catalytic mechanisms and structures are well characterized, their apo-forms can be prepared and reconstituted with Zn(II) salts (A. O. Udom and F. O. Brady, 1980, Biochem. J., 187, 329-335; R. T. Simpson and B.L. Vallee, 1968, Biochemistry, 7, 4343-4349; J. E. Coleman and B. L. Vallee, 1961, J. Biol. Chem., 236, 2244-2249), and they differ in their capacity to bind zinc. Moreover, AP is a dimer containing one catalytic and one co-catalytic zinc atom, as well as one magnesium atom per monomer (W. F. Bosron *et*

al., 1975, Biochemistry, 14, 2275-2282). On the other hand, CPA requires only one zinc atom per molecule to achieve full activity. The distinct differences between the two enzymes are important to rule out specific interactions between MT and either one of them.

The present studies of zinc transfer focus on additional factors that determine its direction. The removal of zinc from zinc enzymes does not occur simply by an interaction with T but requires the participation of other agents. Moreover we have identified groups of agents that drive the zinc acceptor/zinc donor equilibrium in the direction of zinc release.

MATERIALS AND METHODS

Materials. Human and rabbit liver MT-2 were prepared as described (M. Vašák, 1991, Methods Enzymol., 205, 41-44). Rabbit liver Cd_5Zn_2MT-2 was kindly provided by J.H.R. Kägi. Bovine carboxypeptidase A, *E. coli* alkaline phosphatase and chemicals were purchased from Sigma. D.S. Auld provided the CPA substrate dansyl-glycine-glycine-tryptophan (Dns-G-G-W). Deionized water (resistivity of ≥15 MΩ·cm) and metal-free pipet tips (Fisher) were used throughout. Adventitious metals were removed from all buffer stock solutions by treatment with 5 % (v/w) Chelex (BioRad) for two hours at room temperature and subsequent filtration through a Millex-GS micro filter (Millipore).

Preparation of Thionein and Zn_7MT-2. T and zinc substituted Zn_7MT-2 were prepared according to procedures well known in the art (see, W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241). T prepared this way can be stored at -180°C under nitrogen for more than three months without loss of thiol groups. Stock solutions of Zn_7MT-2 were prepared prior to use and excess zinc or Tris removed by 4 to 6 cycles of dilution and concentration with appropriate buffer in a 2 ml Centricon concentrator, molecular weight cutoff = 3,000 (Amicon).

Characterization of Thionein. The reaction of T with DTNB in the absence and presence of different aliquots of zinc sulfate was followed by spectrophotometry at 412 nm in 20 mM Hepes, pH 7.5 at 25°C.

Preparation of apo-alkaline phosphatase. An aliquot of 4 mg AP suspended in 2.5 M ammonium sulfate was transferred to a 1.5 ml Eppendorf microcentrifuge tube. The enzyme was collected by centrifugation and the supernatant discarded. AP was dissolved in

1.5 ml of 10 mM HQSA, pH 8.0, and incubated at 4°C overnight. The sample was transferred to a 2 ml Centricon concentrator (molecular weight cutoff = 30,000) that had been treated with 10 mM HQSA, pH 8.0, concentrated to a final volume of ca. 250 µl, diluted to a volume of 2 ml with chelating agent and re-concentrated, all at 4°C. This procedure was repeated four times. HQSA was then replaced with 10 mM Tris, pH 8.0, and the procedure repeated another 14 times. The enzyme was stored at -20°C. AP and its apo-form were >95 % pure as judged by SDS-PAGE (stacking gel 5 %, resolving gel 12 % acrylamide). Protein concentrations were determined by spectrophotometry ($\epsilon_{278} = 6.77 \times 10^4\ M^{-1}\ cm^{-1}$). The apo-enzyme preparation contained < 3 % of the original zinc and magnesium.

Zinc transfer from Zn_7MT-2 to apo-alkaline phosphatase. Stock solutions of AP and apo-AP in 10 mM Tris, pH 8.0 were diluted with the same buffer to make 0.5 µM working solutions. A solution of 100 mM p-nitrophenyl phosphate in water was diluted with 10 mM Tris, pH 8.0 to obtain a 1 mM substrate working solution. Enzyme (50 µl) and substrate were rapidly mixed in an acid-washed quartz cuvet and the formation of p-nitrophenolate monitored at 400 nm for 2 min (A. O. Udom and F. O. Brady, 1980, Biochem. J., 187, 329-335). A linear best fit was used to calculate the turnover rates between 50 s and 150 s for slow and between 20 s and 60 s for fast reactions. Unless otherwise stated, activity is expressed as percent of that measured for native AP.

Zinc transfer experiments were conducted by pre-incubation of T and AP, or apo-AP with Zn_7MT-2 or Cd_5Zn_2MT-2 and potential agents that enhance transfer in 10 mM Tris, pH 8.0, for two hours at 20°C and subsequent activity analysis.

Preparation of apo-carboxypeptidase A. CPA was purified on a CABS-Sepharose column (L. B. Cueni *et al.*, 1980, Anal. Biochem., 107, 341-349) and stored as a crystal suspension in 10 mM Tris, pH 7.5, at 4°C. Apo-CPA was prepared from CPA crystals (D. S. Auld, 1988, Methods Enzymol., 158, 71-79) and stored as a suspension at 4°C. The purity of CPA and its apo-form was at least 95 % as determined by SDS-PAGE (see above). The zinc:protein ratio for CPA was 1.1 and for the apo-enzyme it was < 0.001. Protein concentrations were determined by spectrophotometry ($\epsilon_{280} = 6.42 \times 10^4\ M^{-1}\ cm^{-1}$).

Zinc transfer from Zn_7MT-2 to apo-carboxypeptidase A. CPA activity was measured by changes in the fluorescence of the substrate Dns-G-G-W (17) with a Biosequential SX-18MV stopped-flow reaction analyzer (Applied Photophysics). Stock

solutions of CPA and apo-CPA, in 20 mM Hepes/1 M NaCl, pH 7.5, were diluted to 2 μM with the same buffer. A stock solution of 20 mM Dns-G-G-W in acetonitrile was prepared and diluted with 20 mM Hepes/1 M NaCl, pH 7.5, to 20 μM.

Activity measurements were carried out at an excitation wavelength of 285 nm (slitwidth 0.3 nm), a detector sensitivity of ca. 400 V and time intervals of 10 ms at 25°C. The emission increase of tryptophan fluorescence was monitored at 340 nm for 50 s. A single exponential best fit was used to calculate the catalytic rates. These were converted into percentages of the rate of native CPA measured under the same experimental conditions.

Zinc transfer experiments were conducted by pre-incubation of apo-CPA with Zn_7MT-2 and potential agents that enhance transfer for defined periods of time at 20°C and subsequent activity analysis. Zinc transfer from CPA to T was measured by incubating the enzyme and T in 1 M Tris, pH 8.0, for 90 min at 20°C and subsequently determining the CPA activity.

Zinc Transfer from Zn_7MT-2 to PAR. MT was incubated with PAR (100 μM) in 20 mM nitrogen-saturated 20 mM Hepes, pH 7.5 and the release of zinc in the absence and presence of sodium selenite and selenocystamine measured by spectrophotometry (ϵ_{500} $(Zn(PAR)_2 = 65{,}000\ M^{-1}cm^{-1}$ (18)) .

RESULTS

Reactivity of MT. a) Zinc transfer from MT-2 to apo-alkaline phosphatase. The activity of a reconstituted zinc enzyme is a measure of the extent of metal transfer from MT-2 to the apo-enzyme. The phosphotransferase activity of the apo-AP preparation used in these experiments was less than 10 % of that of the native enzyme. A 4-fold molar excess of zinc sulfate fully reactivates apo-AP, corresponding to a stoichiometric amount of zinc (four zinc ions, two catalytic and two cocatalytic, per enzyme dimer are required). Zn_7MT-2 also reactivates the apo-enzyme, but somewhat slower than zinc sulfate (>15 min vs. 5 min). However, maximal activation requires more than a 15-fold molar excess of Zn_7MT-2 (Figure 8). Thus, on average, less than one zinc atom is transferred from Zn_7MT-2 to the apo-enzyme.

The extent of reactivation depends on the type of buffer used and its concentration. High concentrations of Tris (above 100 mM as used conventionally for assay of

phosphotransferase activity (A. O. Udom and F. O. Brady, 1980, Biochem. J., 187, 329-335; R. T. Simpson *et al.*, 1968, Biochemistry, 7, 4336-4342; M. Sone *et al.*, 1997, J. Biol. Chem., 272, 6174-6178) and millimolar concentrations of citrate affect the extent of reactivation with MT-2 and zinc sulfate markedly. Increasing the concentration of Tris from 10 mM to 1 M decreases the final phosphatase activity by more than 3-fold, demonstrating competition between Tris and the apo-enzyme for zinc. These effects clearly account for the observation that only 40 % of apo-AP could be reactivated with MT in 1 M Tris, pH 8.0 in previous experiments (A. O. Udom and F. O. Brady, 1980, Biochem. J., 187, 329-335).

Since only about one zinc atom is transferred from MT to the apo-enzyme, the question arises from which cluster and from which particular location in the cluster this zinc atom originates. Therefore, Cd_5Zn_2MT-2 was used as metal donor, since both zinc atoms are located at crystallographically defined positions in the β-domain (A.H. Robbins *et al.*, 1991, J. Mol. Biol., 221, 1269-1293). Reactivation of apo-AP by this MT-2 species, if it occurs, would have to be due to zinc transfer from the β-domain, since cadmium AP, if formed, has only a residual activity of <1 % (J. E. Coleman and P. Gettins, 1983, Adv. Enzymol. Relat. Areas Mol. Biol., 55, 381-480). Cd_5Zn_2MT-2 reactivates apo-AP in a pattern virtually identical to that of Zn_7MT-2 (Figure 8) confirming that zinc is transferred, that it originates in the β-domain, and further indicating that only 1 or 2 zincs are transferred from MT-2. Detailed structural studies of these transfer reactions will be required in order to determine the precise origins and destinations of the zinc ions involved.

b) Enhancement of zinc transfer in the presence of disulfide reagents. Disulfides interact with MT-2 and release zinc ions (*infra*). These reagents also would be expected to enhance zinc transfer from MT-2 to apo-enzymes, hence reactivation of apo-AP in the presence of two such reagents, DTNB and GSSG, was investigated. DTNB is highly reactive and consequently it reacts at a much lower excess and orders of magnitude faster than GSSG (*infra*; T. Y. Li *et al.*, 1981, Biochem. J., 193, 441-446; M. M. Savas *et al.*, 1993, J. Inorg. Biochem., 52, 235-249). Micromolar concentrations of DTNB more than triple the reactivation efficiency of Zn_7MT-2, whereas millimolar concentrations of GSSG are required to achieve the same effect (Figure 9).

c) Zinc transfer from MT-2 to other systems. In order to investigate whether or not these observations apply more generally to the properties of MT in zinc transfer, two other systems were investigated, another apoenzyme and a system that is not based on

measuring zinc transfer by enzymatic assays of zinc enzymes, but on detecting transferred zinc directly. For this purpose we selected the chromophoric zinc chelating agent PAR.

Both zinc sulfate and Zn_7MT-2 reconstitute apo-CPA: with the former, activity is completely restored in a few seconds ($k_f = 7 \times 10^5$ $M^{-1}s^{-1}$ (E. J. Billo *et al.*, 1978, Bioinorg. Chem., 8, 461-475), while with the latter it is slower, requiring more than 30 min to reach completion. Activity increases as a function of the amount of MT-2 added and is restored fully by an equimolar amount of Zn_7MT-2 (Figure 10). Since maximum activity is reached at a stoichiometry of approximately 1:1 (apo-enzyme:MT-2), on average only one zinc atom of MT-2 is transferred. When the transfer reaction is carried out in the presence of 0.1 mM zincon, the absorbance remains unchanged ($\Delta A_{620} \leq 0.005$), indicating that no "free" Zn(II) is released into the medium and that there is no competition between zincon and the apo-enzyme.

We have now shown that MT reacts with a great variety of oxidizing agents and identified cellular agents that can oxidize MT. Among these, the effect of selenium compounds on zinc transfer from MT to PAR is particularly significant. Micromolar concentrations of either selenite or selenocystamine release about four zinc atoms of MT in one hour (Figure 11). The rate and extent of this reaction are comparable to the reaction of MT with DTNB.

Reactivity of thionein: a) zinc transfer from alkaline phosphatase to thionein. We have also begun to study the chemical properties of T, the apoform of MT. There have been almost no studies aside from its capacity to bind zinc apparently owing to its presumed instability. Clearly, T is an important component of the MT system, and the reactivity of its cysteine thiols is a critical parameter for our understanding of the reactivity of the zinc-sulfur bonds in MT. We have now found that T is stable if stored at -180°C in liquid nitrogen and that it can be employed for several hours in biochemical studies without significant oxidation of its thiols. This has allowed us to investigate T in reactions in which it acts as a metal acceptor toward a zinc enzyme, and in redox reactions with disulfides.

The activity of AP depends critically on the concentration of buffer ions. In the presence of Tris, citrate or glutathione (GSH) the catalytic activity of AP decreases over time (Table 5). These agents also influence zinc transfer from AP to T. Addition of T to AP in 10 mM Tris, pH 8.0 only marginally affects the activity of the enzyme but when the identical experiment is performed in 1 M Tris, pH 8.0, AP activity decreases to below 10 % of its original value within approximately 30 min (Figure 12). The concentration of T at which 50 % of the original activity of AP is inhibited (IC_{50}) is about 0.25 μM in 1 M Tris,

pH 8.0. This dramatic decrease in enzymatic activity is completely reversible: Addition of a 200-fold excess of zinc sulfate fully restores its activity. The loss of enzyme activity is likely due to removal of Zn(II) from the active site. Thus, buffer ions like Tris, or cellular agents such as citrate and GSH, clearly mediate zinc transfer. In contrast to Tris, the concentrations at which citrate and GSH are effective are below 1 mM, i.e. in the physiological range.

The effects of T on CPA activity are comparable to those on AP activity: First, addition of T to CPA in 1 M Tris, pH 8.0 progressively decreases the enzyme's activity (data not shown). Second, at substoichiometric amounts of T, the inactivation reaction reaches equilibrium within 30 min (IC_{50} of 0.4 μM). Third, loss of enzyme activity is also most likely due to removal of Zn(II) from the active site since addition of a 100-fold excess of zinc sulfate restores 65 % of the enzyme's original activity.

b) Effect of zinc on the thionein/disulfide interactions. MT reacts with disulfides at neutral pH. For example, T reacts about 30 times faster than MT with DTNB (M. M. Savas *et al.*, 1993, J. Inorg. Biochem., 52, 235-249). Given this difference, it is unclear how zinc influences the reactivity of thiols in this reaction. Therefore, we have determined the reactivity of T toward DTNB as a function of increasing concentrations of zinc. Remarkably, there is a linear decrease of the extent of DTNB reduction to 10% of its original value until four equivalents of zinc are added to T (Figure 13). The remaining reactivity upon adding more zinc corresponds to that of MT at this particular time interval.

DISCUSSION

MT as zinc donor

Several important new aspects of zinc transfer emerge from these studies of the reconstitution of the apo-forms of zinc enzymes by Zn_7MT-2. First of all it should be emphasized that these reactions are thermodynamically unfavorable. The zinc binding constant of T (stability constant of Zn_7MT-2 = 3.2×10^{13} M^{-1} at pH 7.4) and those of the apo-proteins studied (stability constant of AP = 10^7 to 10^8 for both pairs of Zn(II) at pH 6.5 (J. E. Coleman *et al.*, 1982, J. Biol. Chem., 258, 386-395) and of CPA = 2.1×10^8 M^{-1} at pH 8.0 (J. E. Coleman and B. L. Vallee, 1960, J. Biol. Chem., 235, 390-395) predict that zinc transfer in this direction should not be significant (reaction 2).

$$\frac{1}{7}Zn_7MT + P \rightleftharpoons \frac{1}{7}T + ZnP$$

(reaction 2)

However, reaction 2 is based on two assumptions. One is that MT has a metal transfer potential of seven based on cooperative binding of seven zinc ions all with similar binding constants. The other is that the products do not undergo further reactions and thereby shift the equilibrium to the right.

To date, most reports have accepted that zinc binds cooperatively and that only T and Zn_7MT-2 exist without stable intermediates (K. B. Nielson and D. R. Winge, 1983, J. Biol. Chem., 258, 13063-13069). That being so, zinc transfer from MT-2 to apo-enzymes would proceed with the dissociation of all zinc atoms from MT-2 (reaction 3).

$$Zn_7MT + P \longrightarrow T + 6Zn(II) + ZnP$$

(reaction 3)

Neither of these reactions seem to occur in the manner indicated. Thus, the stability constant of Zn_7MT-2 is too high to account for zinc transfer from a thermodynamically relatively stable system (MT-2) to others that are orders of magnitude more labile (CPA, AP) under stoichiometric concentrations of the reactants. If the binding constants were the same for all seven zinc atoms in MT-2, only a negligible fraction of zinc should be transferred. Indeed, without a driving force, the occurrence of zinc transfer would be a surprising result. The observed metal transfer potential of MT-2 is approximately one in the case of CPA and perhaps less than that in the case of AP. Since addition of zincon to this reaction did not detect any increase in the pool of free zinc, transfer does not induce further dissociation of the zinc as postulated by reaction 3. Therefore formation of T during the transfer reaction can be ruled out. Yet, six of the seven zinc ions in the system have to be accounted for. Quite possibly, they initially remain bound as a Zn_6MT-2 species. At present, the precise structure of this species is unknown. Rearrangement of Zn_6MT-2, perhaps with the formation of two Zn_3 clusters, might occur since the coordination of MT is quite flexible as demonstrated for copper MT where an MT species containing one Cu_6S_9 and one Cu_6S_{11} unit has been reported (A. Presta *et al.*, 1995, Eur. J. Biochem., 227, 226-240). MT exhibits high structural flexibility, and cadmium exchange inside the β-cluster and between the β-clusters of two MT molecules is fast (J. D. Otvos *et al.*, 1993, in Metallothionein III, eds. Suzuki, K. T., Imura, N. & Kimura, M. (Birkhäuser, Basel),

57-74). A second possibility is that the β-cluster is stabilized through extensive delocalization of zinc as is known for cadmium in a Cd_6MT species obtained after treating Cd_7MT-2 with EDTA (F. Vazquez and M. Vašák, 1988, Biochem. J., 253, 611-614). In the absence of any enhancers (see below), such types of rearrangements indeed might be not only the rate-determining step in the zinc transfer reaction - the simple uptake of zinc ions by both apo-enzymes studied is faster than the transfer reaction - but also the driving force that brings about this thermodynamically unfavorable process. Removing Zn_6MT-2 from the equilibrium with Zn_7MT-2 would indeed explain why zinc can be released from the latter at all (reaction 4). Thiol oxidation might be yet another mechanism facilitating this process since this would decrease ligand strength. In particular, we have found that the uncoordinated thiols in T react much faster with oxidizing agents such as DTNB than do the metal-bound thiols in MT-2 and that this reactivity is modulated by zinc (Figure 13).

$$Zn_7MT + P \longrightarrow Zn_6MT + ZnP \longrightarrow Zn_6MT\text{(oxidized/isomerized)}$$

(reaction 4)

The transfer of a single zinc ion from Zn_7MT-2 raises the question why MT-2 complexes seven zinc ions but transfers only one. Since Zn(II) binding to apo-CPA (24) or apo-AP (9) is either comparable to or faster than zinc transfer from MT-2 to these apo-enzymes, processes involved in the release of ionic zinc from MT-2 seem to contain the rate-determining step in the transfer reaction. On a physiological timescale non-enhanced transfer appears rather slow, hence it seems likely that the rate and extent of zinc release from MT-2 should be enhanced *in vivo*. The unique structure of MT offers several possibilities of how such enhancement could be achieved *in vitro*.

One way to release zinc ions from Zn_7MT-2 would be by displacing them with different metal ions that can form tighter complexes with MT-2. Interestingly, zinc is orders of magnitude less tightly bound to T than for example Cd(II) or Cu(I) (J. H. R. Kägi, 1993, in Metallothionein III, eds. Suzuki, K. T., Imura, N. & Kimura, M. (Birkhäuser, Basel), 29-55). Cd(II) or Cu(I) replace zinc in MT-2 within seconds. Therefore, metal replacement reactions could constitute a means by which more than one zinc atom from Zn_7MT-2 is made available to activate apo-proteins. Owing to the unusually high binding constant of Cd_7MT-2 in coordination sites that contain thiolate sulfur ($K_{Cd} = 3.2 \times 10^{17}\ M^{-1}$ at pH 7.4 (29)) Cd(II) will preferentially replace Zn(II) in MT-2 instead of binding to nitrogen and oxygen ligands in enzyme sites. The release of one zinc ion by metal-exchange requires one cadmium or cuprous ion. Since in most organisms both cadmium and copper are far less

abundant than zinc the likely physiological importance of this metal-exchange observed *in vitro* remains obscure.

Thiol modification reactions are particularly attractive as a means to release zinc since they affect the zinc ligands of MT (Fliss, H. & Ménard, M. (1992) Arch. Biochem. Biophys. 293, 195-199; W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241) and are biochemically feasible. Such reactions, possibly involving cellular GSSG, would not only facilitate release but also increase the number of zinc ions transferred per MT molecule (with the thiolation reaction as the rate-determining step) *in vivo*. From a range of possible transfer enhancing reagents two thiol/disulfide-exchange reagents, DTNB and GSSG, were investigated here as examples of disulfides with greatly differing reactivities. DTNB is a highly reactive thiolation agent. It rapidly thiolates the sulfhydryl groups of MT-2, with concomitant release of ionic zinc. Moreover, it significantly increases the number of zinc ions transferred per MT-2 molecule. The reactivation of apo-CPA or -AP in the presence of micromolar concentrations of DTNB is more than three times as great as in its absence, indicating that only three to four, but not all seven zinc ions become available for transfer. GSSG has similar effects, although it requires millimolar concentrations to achieve the same enhancement. Obviously the lower reactivity of GSSG toward MT-2 (W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241) as compared with DTNB (T. Y. Li *et al.*, 1981, Biochem. J., 193, 441-446; M. M. Savas *et al.*, 1993, J. Inorg. Biochem., 52, 235-249) affects the rate of zinc release and consequently the rate of zinc uptake of the apo-enzyme on the timescale of the experiment. In the case of CPA, DTNB also increases the extent of zinc transfer while no clear effect was seen with GSSG up to 1 mM. In addition, we have now identified selenium compounds as possible agents that increase the rate of zinc transfer from MT to an acceptor at micromolar concentrations of reagents. Selenium is an essential trace element that is involved in the regulation and maintenance of the cellular thiol redox state. Our experiments indicate that zinc transfer reactions may well be a possible target for the molecular action of selenium.

T as a zinc acceptor

In the absence of a driving force for the forward or backward reaction, the equilibrium of removal of zinc from a zinc protein by T (reaction 5) should be controlled thermodynamically. Since for most proteins the binding constant for zinc is at least 1,000 fold lower than that for T, zinc transfer from zinc enzymes to T should be preferred thermodynamically.

$$\text{Zn-P} + \text{T} \rightleftharpoons \text{Zn-MT} + \text{P}$$

(P = apo-protein)

(reaction 5)

In the case of AP, however, despite expectations based on the relative stability constants of MT and AP, T does not appear to sequester significant amounts of zinc from the active site of the enzyme. Thus, T does not necessarily act as a strong chelating agent toward enzymes. If this were so, then the induction of T in the cell might interfere with many zinc-dependent enzymatic processes. However, T does inactivate AP in the presence of buffer ions such as Tris or citrate. One possible explanation is that the buffer ions help deplete the enzyme of its zinc, and thereby act to shuttle zinc between the enzyme and T. The fact that T is ineffective in 10 mM Tris strongly suggests that there is no direct interaction of T and the zinc enzyme. Nor is a specific inhibitory effect of T very plausible, since T acts in substoichiometric amounts and also inactivates CPA, mitigating against a specific AP-T interaction. In effect, Tris and citrate lower the stability constant of AP. Tris is not only a buffer ion, but also a co-substrate for AP (M. Sone *et al.*, 1997, J. Biol. Chem., 272, 6174-6178; J. E. Coleman and P. Gettins, 1983, Adv. Enzymol. Relat. Areas Mol. Biol., 55, 381-4801) and a chelating agent. Zinc chelating agents such as citrate and GSH occur in the cytosol in millimolar concentrations (A. Meister, 1988, J. Biol. Chem., 263, 17205-17208; N. Westergaard *et al.*, 1995, Proc. Natl. Acad. Sci. USA, 92, 3367-3370; A. F. Dulhunty, 1988, Biophys. J., 53, 609-616) and could serve such a purpose (reaction 6). These agents do not release zinc from MT, presumably because they cannot gain access to the zinc ions in the clusters. They are important determinants for the direction of zinc transfer. Under the conditions investigated here they favor the unidirectional transfer of zinc from AP to T.

$$\text{Zn-P} + \text{T} + \text{SA} \rightleftharpoons \text{Zn-SA} + \text{T} + \text{P} \longrightarrow \text{Zn-T} + \text{SA} + \text{P}$$

(SA = shuttle agent)

(reaction 6)

The concentrations of both citrate and GSH can vary over a relatively wide range in the cell (A. Meister, 1988, J. Biol. Chem., 263, 17205-17208; N. Westergaard *et al.*, 1995, Proc. Natl. Acad. Sci. USA, 92, 3367-3370; A. F. Dulhunty, 1988, Biophys. J., 53, 609-616). It is, therefore, conceivable that such changes of concentrations affect the direction of zinc transfer *in vivo* and could render T a specific chelating agent under yet to be defined

circumstances. Moreover, T is a very strong reductant. It reacts significantly faster with disulfides than does MT. The reactivity of T toward disulfides and other oxidizing agents is efficiently quenched in the presence of zinc (Figure 13). Whether or not such reducing power of T is needed in the cell and whether or not such reducing power, or as a matter of fact the reductive functions of any cellular thiols, are controlled by binding of zinc is currently unknown.

Implications: Our results suggest that zinc transfer between zinc enzymes and MT should not be considered a simple two-component system as earlier interpretations suggested (A. O. Udom and F. O. Brady, 1980, Biochem. J., 187, 329-335). We have already found two types of agents that modulate the release of zinc from its thermodynamically tight binding sites in the clusters of MT (oxidizing agents) and that carry zinc between MT and a participating protein (zinc chelating agents). We have shown that GSSG releases zinc, but we also find that certain buffers like Tris and citrate chelate metals including zinc, and, under certain circumstances form complexes that significantly affect the state of zinc and have a marked influence on metal transfer. In fact, they might become the cellular factors that form intermediates which could enhance or control transfer. As such they could participate in the conveyance of zinc from MT to other proteins and in the mechanism of transfer. Zinc is always associated with another cellular ligand and is not transferred as the free zinc ion but in the form of a complex with any one or several of these agents that together constitute a distribution system. Clearly, the number and identities of such agents are unknown at this point, as are the details and extent to which they would be sensitive to the oxidative and reductive state of the environment for properties of MT as a zinc donor or zinc acceptor. The nature and identity of the shuttle and distribution system is currently under investigation.

Table 5: Effect of buffers and chelating agents on the inactivation of AP by T.

Agent added*	[Agent], mM	AP Activity in the Absence of T, %	AP Activity in the Presence of T, %
none	0	100	> 80
Tris	500	90.8	11.5
Citrate	0.5	94	40.4
GSH	0.5	94.8	77.2

*AP, 0.5 μM, was incubated with T, 1 μM, in 10 mM Tris, pH 8.0 in the presence of Tris, sodium citrate, or GSH. Aliquots were taken after two hours and assayed spectrophotometrically for enzymatic activity.

6.3. <u>THIOLATE LIGANDS IN METALLOTHIONEIN CONFER REDOX ACTIVITY ON ZINC CLUSTERS</u>

The interplay between redox-inert zinc and the redox-active thiol of cysteine has emerged as a general biological principle which endows the stable zinc binding sites in proteins with dynamic functions. Metallothionein (MT) is a key protein in this regard: it binds and releases zinc in a range of redox potentials readily achieved in the cell. We have identified reagents that can oxidize the zinc-sulfur clusters in MT and thereby mobilize zinc in the cell, and we propose the position of MT on a chemical redox scale. Our data support the hypothesis that the function of MT, which has been sought for 40 years, resides in the coordination structure of its clusters and the redox activity of their sulfur ligands. This function likely controls and distributes zinc in the cell. Its function in this regard is specific and mitigates the idea that MT is a scavenger of radicals or a detoxifier of heavy metals. It seems unlikely that MT would be primarily a site of sequestration of toxic metals in the cell, since the redox reactivity of its cysteine ligands would render its heavy metal complexes similarly susceptible to mobilization by oxidative reactions. The biological significance of the widespread use of cysteine as a ligand of zinc in proteins would seem to reside in the capacity of thiols to bind zinc very tightly *and* of oxidized thiols to bind it weakly. This concept integrates biologically redox-inert zinc, not only in MT but possibly in zinc finger or other proteins, into the redox chemistry of the cell and suggests a new mechanism of cellular control, in which a high reducing power is related to zinc binding while a low reducing power is related to zinc mobilization.

Metallothionein (MT) is a unique metalloprotein in which cysteine constitutes one third of its amino acids and histidine and aromatic amino acids are absent. All twenty cysteines bind zinc such that each metal atom has a complement of four cysteine ligands. The zinc atoms are arranged into two discrete clusters each with bridging cysteines. In one cluster nine cysteines bind three zincs, and in the other eleven bind four. This constellation, unique so far, is encountered solely in MTs. The physiology of MT has remained unknown and its three-dimensional structure was established only less than a decade ago ((M^+)

We have performed and advocated experiments to relate the structure of MT to its possible function(s) on the basis of the nature of zinc coordination in MT (B. L. Vallee, 1979, Experientia Suppl., 34, 19-40; B. L. Vallee, 1987, Experientia Suppl., 52, 5-16; B. L. Vallee and W. Maret, 1993, in Metallothionein III, eds. Suzuki, K. T., Imura, N., & Kimura, M. (Birkhäuser, Basel), 1-27). We have suggested that the characteristics of the cluster motif might be the key to the mode of cellular zinc distribution (B. L. Vallee, 1991, Meth. Enzymol., 205, 3-7). MT binds zinc with high thermodynamic stability [$K_D = 1.4 \times 10^{-13}$ M for human MT at pH 7.0 (J. H. R. Kägi, 1993, in Metallothionein III, eds. Suzuki, K. T., Imura, N., & Kimura, M. (Birkhäuser, Basel), 29-55)] while simultaneously providing a mechanism for kinetic lability whereby zinc can be released at rates that are orders of magnitude greater than those observed for zinc metalloenzymes. Zinc as well as cadmium are known to undergo rapid inter- and intracluster exchange (Otvos, J. D., Liu, X., Li, H., Shen, G., & Basti, M. (1993) in Metallothionein III, eds. Suzuki, K. T., Imura, N., & Kimura, M. (Birkhäuser, Basel), pp. 57-74; W. Maret *et al.*, 1997, Proc. Natl. Acad. Sci. USA, 94, 2233-2237).

MT apparently binds zinc with higher affinity than do many other proteins. For MT to serve as a source for the distribution of zinc, mechanisms would be required that could regulate the binding and release of the metal. It has been shown that an interaction of MT with glutathione disulfide or other biological disulfides releases zinc (W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241; W. Maret, 1995, Neurochem. Int., 7, 111-117) and that the combination of glutathione *and* glutathione disulfide enhances transfer of zinc from MT to an apoenzyme (*infra*). This has led us to infer that the reactivity and redox behavior of the sulfur ligands in the MT clusters are crucial for the dynamic state of zinc. We proposed that the zinc sulfur-cluster chemistry might be sensitive to changes of the cellular redox state and that oxidizing conditions induce the transfer of zinc from its binding sites in MT to those of lower affinity in other proteins.

We here provide additional support for this concept and show that a number of compounds including some of potential biological importance can oxidize the thiolate

ligands of zinc in MT with concomitant release of zinc. We conclude that the redox properties of MT and their effect on zinc in the clusters are crucial to its function which is further affected by additional interactions (*infra*).

MATERIALS AND METHODS

Materials. Charge-separable cadmium and zinc MT-1 and -2 isoforms were prepared from rabbit or human liver according to established procedures (M. Vašák, 1991, Meth. Enzymol., 205, 41-44). DsbA was from Boehringer Mannheim. The sources of other chemicals are well known to those in the art (see, W. Maret *et al.*, 1997, Proc. Natl. Acad. Sci. USA, 94, 2233-2237).

Preparation of metallothionein isoforms. MT was reduced and reconstituted with zinc to obtain species that have i) a defined content of 20 sulfhydryls as determined by spectrophotometric titrations with 2,2'-dithiodipyridine (ϵ_{343} = 7,600 $M^{-1}cm^{-1}$), and ii) a stoichiometry of seven zinc per molecule as determined by atomic absorption spectrophotometry with a Perkin-Elmer Model 2280 instrument. Protein concentrations of MT were determined by spectrophotometry [ϵ_{220} = 155,000 $M^{-1}cm^{-1}$ (A. Schäffer, 1991, Meth. Enzymol., 205, 529-540)].

Spectrophotometric assays. Reactions between MT and oxidizing agents were monitored by two types of assays. One assay is based on measuring zinc release from MT with the zinc-complexing dyes 4-(2-pyridylazo)resorcinol (PAR), ϵ_{500} = 65,000 $M^{-1}cm^{-1}$ (C. F. Shaw III *et al.*, 1990, Inorg. Chem. 29, 403-411; H. Fliss and M. Ménard, 1992, Arch. Biochem. Biophys., 293, 195-199) or 2-carboxyl-2'-hydroxy-5'-sulfoformazylbenzene (zincon), ϵ_{620} = 17,500 $M^{-1}cm^{-1}$. The other assay is based on the reduction of oxidizing agents with suitable chromophoric properties. Redox reactions were measured at 25 °C with a Cary 1E spectrophotometer (Varian) by following the reduction of the oxidizing agent at its absorption maximum.

RESULTS AND DISCUSSION

The Zn-S bond of MT clusters underlies their functions. Classically zinc has not been thought to be associated with biological redox reactions, since the metal is not readily

oxidizable or reducible in solution. However, it is a distinct possibility that a change in the redox status of a donor atom of a zinc compound might alter the overall oxidoreductive properties of the complex and hence its biological reactivity. In MT, four thiolates interact with each of the seven zinc ions to form two clusters. The stability of zinc binding in these clusters could be altered selectively through interactions with cellular oxidoreductants. As a consequence, individual zinc atoms in the clusters could be differentially affected and become particularly reactive for transfer to other proteins. It is unlikely that such possibilities would have been considered prior to the determination of the MT structure, nor would one have been led to postulate or anticipate that a cluster structure might be a chemical basis for storage, release, or exchange of zinc. Indeed we are unaware that the oxidoreductive state of the donor atom(s) of a zinc complex - be it sulfur, nitrogen, or oxygen - has been postulated to feature in biological zinc complexes of proteins and to affect function. This hypothesis suggests an important general principle that could underlie similar interactions in other biological compounds whose presumable kinetic and thermodynamic behavior would also not be predictable. The MT cluster structure which allows zinc to bind tightly and stably but to become mobile through donor atom modification could have far reaching implications. The manner and extent of such changes, brought about in metabolism, would become the subject of intensive investigations in numerous enzymatic and molecular and cellular biological systems now known. We ourselves are examining a number of these to define as far as possible redox partners for the zinc-sulfur cluster components and the number and identity of the zinc atoms in the clusters involved.

Table 6. Reactions of Metallothionein with Oxidizing Agents

Redox Reagent (mV)	n^*	Redox Potential†	Reaction with MT‡	References
H_2O_2/H_2O	2	+1776 E_o	++	A. R. Quesada, 1996, Arch. Biochem. Biophys. 334, 241-250.
HO_2/H_2O_2	1	+1495 E_o	++	P. J. Thornalley and M Vašák, 1985, Biochim. Biophys. Acta, 827, 36-44.
HNO_2/NO	1	+ 983 E_o	++	K.-D Kröncke *et al.*, 1994, Biochem. Biophys. Res. Commun., 200, 1105-1110.
ferricinium/ferrocene	1	+ 400 E_o	++	here
ferricyanide/ferrocyanide	1	+ 358 E_o'	++	here; C. Chen *et al.*, 1996, Biochem. J., 317, 389-394.
azurin (*P. aeruginosa*) (Cu(II)/Cu(I))	1	+ 330 E_o'	+	here
Wurster's Blue§	1	+ 260 E_o'	+	here
cytochrome c (Fe(III)/Fe(II))	1	+ 254 E_o'	+	here; C. Simpkins *et al.*, 1993, Life Sci., 53, 1975-1980.
ascorbate/ dehydroascorbate	1 or 2	+ 58 E_o'	+	here
selenate/selenite	2	+ 50 E_o	++	here
$DsbA_{ox}/DsbA_{red}$ (*E. coli*)	2	- 125 E_o'	+	here
dithionitrobenzoic acid/ thionitrobenzoic acid	2	- 150 E_o'	++	M. M. Savas, 1993, J. Inorg. Biochem., 52, 235-249.
$FAD/FADH_2$	2	- 220 E_o'	no	here

Table 6. Reactions of Metallothionein with Oxidizing Agents

Redox Reagent (mV)	n*	Redox Potential†	Reaction with MT‡	References
glutathione disulfide/ glutathione	2	- 240 E_o'	+	W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241; W. Maret, 1995, Neurochem. Int., 7, 111-117.
coenzyme A disulfide/ coenzyme A	2	- 248 E_o'	+	W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241; W. Maret, 1995, Neurochem. Int., 7, 111-117.
cystamine/cysteamine	2	- 251 E_o'	+	W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241; W. Maret, 1995, Neurochem. Int., 7, 111-117.
NAD^+/NADH	2	- 320 E_o'	no	here
selenite/selenium	4	- 366 E_o	++	here
methyl viologen	1 or 2	- 440 E_o'	no	here

* number of electrons
† refs. 18-20
‡ The reactions are characterized in terms of their half-lives; ++ indicates minutes to one hour; + indicates longer than one hour
§ N,N,N',N'-tetramethyl-1,4-phenylenediamine

The reaction of oxidizing agents with MT and definition of properties of MT as a redox-active protein. Table 6 shows that MT is oxidized by a large number of agents whose redox potentials cover a wide range. Iron(III) (ferricyanide, cytochrome c, ferricinium ion) and copper(II) compounds, ascorbate, and disulfides are examples of such redox couples. The agents are tabulated in terms of their suitability to i) estimate the redox potential of MT, ii) determine the lowest redox potential with which MT could be oxidized, and iii) serve as possible partners that might oxidize MT *in vivo*.

We have so far examined the reaction of MT with disulfides and cytochrome c in particular detail. Glutathione (GSH) inhibits zinc release from MT (*infra*), but it enhances the glutathione disulfide (GSSG)-induced release of zinc from MT (Figure 15). This is in agreement with our data that show modulation of zinc transfer from MT to zinc-depleted sorbitol dehydrogenase by GSH and GSSG (*infra*). Based on changes of the Soret band of ferricytochrome c, it has been concluded recently that rabbit liver Cd,Zn-MT reduces cytochrome c (C. Simpkins *et al.*, 1993, Life Sci., 53, 1975-1980), but it was unknown whether or not zinc is released in this process. Indeed, human liver MT-2 is oxidized by cytochrome c with concomitant release of zinc (Figure 16). In some cases it is possible to monitor both reduction of the oxidant and zinc transfer simultaneously to ascertain that redox reactions are indeed coupled with the release of zinc. Thus, when MT reacts with the disulfide dithiodipyridine, all thiols of MT are oxidized after one hour and concomitantly seven zinc ions are released as detected by reaction with zincon (Figure 17; Zincon ($K_{D(Zn)}$ = 1.26×10^{-5} M) is the only suitable chromophoric reagent known to us for the purpose, since it does not react with MT and, hence does not affect the reactivity of thiols in MT).

Thus far disulfides and selenite have proved to have the lowest redox potentials to oxidize MT. These findings provide an upper limit for the redox potential of MT, which must be below -366 mV, i.e. sufficiently low to allow cellular oxidants to react with MT and release zinc. A redox potential of MT in a range of < -366 mV would also be consistent with direct electrochemical studies on MT (R. W. Olafson, 1988, Bioelectrochem. Bioenerg. 19, 111-125), in which cyclic voltammetry has revealed oxidation/reduction waves of MT around -380 mV (-600 mV against Ag/AgCl). Since in biology the differences of potentials between redox couples are relatively small, disulfides and selenite are the most likely candidates for the oxidation of MT *in vivo*. The potentials given in Table 6 are midpoint potentials in which the concentration of the oxidant is equal to that of the reductant, a situation rarely encountered in the cell. In fact, it is not uncommon for energy-dependent mechanisms to hold redox couples such as thiol/disulfide pairs in a nonequilibrium state (H.

F. Gilbert, 1995, Meth. Enzymol., 251, 8-28). Thus, for the glutathione state, the redox potential derived from measured GSH/GSSG ratios of 30:1 to 100:1 in the cytosol has been calculated to fall between -221 and -236 mV (C. Hwang *et al.*, 1992, Science, 257, 1496-1502). In the endoplasmic reticulum, however, the GSH/GSSG ratio is in the range from 1:1 to 3:1 (C. Hwang *et al.*, 1992, Science, 257, 1496-1502) corresponding to redox potentials of -133 to -165 mV, respectively (for a total glutathione concentration of 1 mM). Thus, an oxidizing power stronger than indicated by the midpoint potential can be attained, particularly in a compartmentalized redox environment.

The capacity of biological disulfides to release zinc from MT varies considerably. Cystamine e.g. releases 93% of zinc from MT in one hour while in the same time and at the same concentration glutathione disulfide releases only 20% (W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241; W. Maret, 1995, Neurochem. Int., 7, 111-117). Yet, the identity of biological disulfides that might react with MT *in vivo* is not known with certainty. We speculated that "the physiological reactant could well be a disulfide other than GSSG or a particular disulfide of a given protein or proteins" and that "if GSSG indeed is the cellular disulfide involved, then its reaction with MT might be enzyme catalyzed" (W. Maret, 1994, Proc. Natl. Acad. Sci. USA, 91, 237-241). Since then we have examined protein disulfides as possible candidates. The family of thiol/disulfide oxidoreductases includes thioredoxins and protein disulfide isomerases; we have selected DsbA for study as it has the highest redox potential (-125 mV) (F. Åslund *et al.*, 1997, J. Biol. Chem., 272, 30780-30786). Indeed, stoichiometric amounts of this enzyme react with MT and increase zinc release (Figure 18). This experiment suggests that release of zinc from MT in the cell also could occur through a specific process generating a protein disulfide, presumably as a result of a local signaling event, and obviating associated concerns regarding overall redox changes in the whole cell. While a considerable change of the cellular redox state could affect many other processes, the localized production of a protein disulfide would provide the specificity that would be needed for zinc release.

Redox reactions of zinc-coordinated thiolate ligands and the state of sulfur in oxidized MT. One-, two-, and four-electron oxidants react with MT (Table 6). Disulfides; thiyl radicals, which can dimerize to disulfides; or, in the presence of oxygen, sulfenic acids (-SOH), sulfinic acids (-SO_2H) and sulfonic acids (-SO_3H) are possible oxidation products of thiols. The last have been generated by the oxidation of coordinated thiolate ligands (C. G. Kuehn and S. S. Isied, 1980, Prog. Inorg. Chem., 27, 153-221), though we are unaware that such oxidation products including disulfides can act as metal ligands in a biological zinc

complex. Sulfenic acid derivatives of cysteine, however, have been identified in a few enzymes either as stable or functional residues (A. Claiborne *et al.*, 1993, FASEB J,. 7, 1483-1490). Their stabilization requires the absence of other vicinal thiols. The presence of twenty thiols in MT, therefore, would not favor their existence. Formation of thio acids would change the overall charge of MT. We have found no gel electrophoretic evidence for altered charges in oxidized MT (data not shown). MT oxidation products generated with the hydroxyl radical or superoxide anions can be fully reduced to the native protein (P. J. Thornalley and M Vašák, 1985, Biochim. Biophys. Acta, 827, 36-44). This is generally taken as proof that oxidation states higher than disulfides have not formed though this assumption is in need of reexamination and extension in the light of the present considerations. Moreover, MT reacts with nitric oxide to form disulfides, as demonstrated directly by resonance Raman spectroscopy (K.-D Kröncke *et al.*, 1994, Biochem. Biophys. Res. Commun., 200, 1105-1110). Mixed disulfides and intra- and intermolecular disulfides are thought to be the product of oxidation of MT with disulfides such as Ellman's reagent (M. M. Savas, 1993, J. Inorg. Biochem., 52, 235-249) or with ferricyanide (C. Chen *et al.*, 1996, Biochem. J., 317, 389-394). Also, the reaction of selenite with thiols results in the production of disulfides (R. Gopalakrishna *et al.*, 1997, Arch. Biochem. Biophys., 348, 25-36). Only hydrogen peroxide oxidizes thiols in MT to levels higher than disulfides (A. R. Quesada, 1996, Arch. Biochem. Biophys. 334, 241-250). Thus, most agents listed in the table oxidize thiolate sulfur in MT to disulfides. Based on current knowledge this oxidation state would seem to be the most attractive biologically since it is readily reversible to the thiol state. Two structural features of MT strongly favor the formation of disulfides, i.e. the Cys-Cys and Cys-X-X-Cys motifs in the linear protein sequence and the juxtaposition of thiols in the three-dimensional structure. The cluster structure could thereby contain the blueprint for the formation of specific disulfides that might influence the specificity of zinc release. The magnitude and number of interrelationships between such potential disulfides formed among twenty cysteine containing segments cannot be predicted from the linear sequence of MT. Their establishment and possible interaction in MT would almost certainly turn out to be a formidable experimental task by techniques now feasible.

Certainly the reactivities of different thiol/disulfide couples in MT would vary significantly, since their stability is influenced to a large extent by steric effects that can lead to differences of eleven orders of magnitude in equilibrium constants, equivalent to differences of 330 mV in their redox potential (H. F. Gilbert, 1995, Meth. Enzymol., 251, 8-28).

Corollaries of the redox properties of MT. Establishing the redox potential of MT that allows it to be readily oxidized by cellular constituents not only provides a different approach to determine its function, but beyond that, gives a new perspective to its nature. MT is known to react with radicals and reactive species and has been thought to be an antioxidant (M. Sato and I. Bremner, 1993, Free Radic. Biol. Med., 14, 325-337). This potential is entirely consistent with its ranking as a relatively strong reducing agent on the redox scale (Table 6). Indeed, MT is a chemically reactive molecule and should interact with a large number of agents. If scavenging of reactive species were a physiological function of MT, its capacity to release zinc and the subsequent cellular effects must be accounted for. We believe that MT has specific redox properties for a purpose which selectively controls the release and uptake of zinc rather than being a nonspecific antioxidant that releases the metal randomly and sporadically. Interestingly, the redox properties of MT also raise significant questions about the putative role of MT as a detoxifier of heavy metals. The relatively high reactivity of MT can also cause the dissociation of heavy metals as has now been shown for nitric oxide-induced release of cadmium from MT (R. R. Misra *et al.*, 1996, Chem. Res. Toxicol., 9, 326-332). This observation must be somewhat disturbing to advocates of the concept that its primary function is to protect living matter against the consequences of metal pollution. Such findings can hardly be considered as support for the belief that MT constitutes a safe repository against pollution with unwelcome heavy metals.

Retrospects and Prospects. The complete absence of aromatic amino acids or any other components with prominent spectroscopic features including the optically silent cadmium and zinc atoms hampered the identification of MT as a protein, its isolation, characterization, localization, and recognition of its metabolic role(s). More than thirty years passed between its discovery (M. Margoshes and B. L. Vallee, 1957, J. Am. Chem. Soc., 79, 4813-4814) and the establishment of its three-dimensional structure (A. H. Robbins *et al.*, 1991, J. Mol. Biol., 221, 1269-1293; K. Wüthrich, 1991 Meth. Enzymol., 205, 502-520). Even the latter achievement failed to provide parameters to guide the experimental search for its function.

It was during these thirty years that the indispensability of zinc to development, growth and differentiation in all phyla and species gained prominence together with the recognition of zinc-binding signature sequences in enzymes and other proteins that underscore zinc's biochemical and functional diversity (B. L Vallee and K. H. Falchuk, 1993, Physiol. Rev., 73, 79-118). The identification of MT as an oxidoreductive component

of a zinc storage and distribution system simultaneously raises questions of how it might operate to ensure homeostasis and fine-tuning. Much as the important and potentially far reaching role of MT in this regard has just been recognized, the emerging knowledge of zinc metabolism is provocative and timely and suggests the existence of a corollary metabolic system at the intersections of protein, nucleic acid and zinc chemistry. Clearly, the distribution of zinc and the priorities of its delivery and transfer requires as careful an orchestration and coordination as that of the macromolecules to which it lends specificity. It seems reasonable to postulate the existence of a regulatory system at these interfaces designed to achieve fine-tuning of the relevant processes.

The awareness of the importance of zinc in metabolism is counterbalanced by the large gaps in knowledge of the laws that regulate its participation and how they relate to MT. Clearly, zinc binds quite tightly to proteins and hence, free zinc is not readily available in the cell. Rather, a network of proteins seems to coordinate its trans-membrane traffic (R. D. Palmiter and S. D. Findley, 1995, EMBO J., 14, 639-649; R. D. Palmiter *et al.*, 1996, EMBO J., 15, 1784-1791; R. D. Palmiter *et al.*, 1996, Proc. Natl. Acad. Sci. USA, 93, 14934-14939; D. Eide, 1996, Curr. Opin. Cell Biol., 9, 573-577; H. Gunshin *et al.*, 1997, Nature, 388, 482-488; L. Huang and J. Gitschier, 1998, Nature Genet., 17, 292-297) at considerable expenditure of energy, apparently ensuring and safeguarding its availability, and to provide it when needed. If zinc is handled almost exclusively while complexed to proteins, how is it then passed from one protein to another and how is it distributed and allocated? Answers to such questions are not obvious, since zinc traffic does not seem to be driven solely by thermodynamic gradients: flux of zinc does not necessarily move it from a site of low to others of higher binding affinity. Here MT exemplifies how zinc is handled. In MT, zinc is also bound quite tightly with an overall dissociation constant of 1.4×10^{-13} M at pH 7.0 (J. H. R. Kägi, 1993, in Metallothionein III, eds. Suzuki, K. T., Imura, N., & Kimura, M. (Birkhäuser, Basel), 29-55), i.e. one of the tightest binding constants of zinc in the cell. How then can zinc be transferred from MT to binding sites of lower binding affinity? Our results suggest how this could be achieved.

The mechanisms by which zinc is mobilized from its biological binding sites and distributed among proteins define an important aspect of inorganic biochemistry. While thiol redox chemistry apparently controls the flux of redox-inert zinc, the strong binding of zinc to thiol ligands also suggests that redox-inert zinc might help to control the thiol/disulfide redox state much as this influence of zinc on redox equilibria has not been widely appreciated. The gap in knowledge has apparently persisted owing to the fact that the properties of zinc have held little attraction for experimentalists since the metal lacks

accessible spectroscopic properties and, because much of the zinc in the typical adult is contained in MT, a protein whose function was not apparent.

6.4. CONTROL OF ZINC HOMEOSTASIS BY ADMINISTRATION OF EBSELEN AND OTHER SELENIUM COMPOUNDS

Selenium compounds are preferred as oxidizing and reducing agents for control of zinc release from metallothionein. Non-limiting examples of such preferred selenium compounds include as oxidizing agents, ebselen, selenocystine, selenocystamine, selenoglutathione, and as reducing agents, selenocysteine, selenoglutathione, selenocysteamine.

Ebselen is a preferred oxidizing agent for the release of zinc from metallothionein in the present invention. The ability of ebselen to release zinc from metallothionein has been demonstrated as described in the following sections.

6.4.1. EBSELEN, A SELENIUM CONTAINING DRUG, RELEASES ZINC FROM METALLOTHIONEIN

Selenium compounds oxidize the thiolate ligands in the zinc clusters of metallothionein and release zinc. This hitherto unrecognized chemistry defines new cellular targets for selenium compounds and suggests important interactions between the two essential elements zinc and selenium. In attempts to delineate further the redox chemistry of biological zinc complexes with thiolate ligands, we found that the selenium-containing drug ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) releases zinc from metallothionein. The reaction is very rapid ($t_{1/2} < 1$ min) and proceeds through opening of the isoselenazol ring and formation of a selenenyl sulfide with metallothionein. In contrast, the reaction with glutathione is even faster ($t_{1/2} < 1$ s) and first forms a selenol and then a diselenide derivative of ebselen. The reaction with metallothionein is stoichiometric and not affected by hydrogen peroxide. Hence, ebselen is not a glutathione peroxidase mimic in this reaction. Yet, it illustrates the functional potential of active site selenocysteines in selenoproteins in general. These findings reveal a hitherto unknown mode of action for this non-toxic experimental drug and therefore suggest new therapeutic applications in zinc-related medical disorders.

The biological oxidizing agents that induce zinc release from MT feature prominently disulfides, selenium salts, and selenocystamine. Unlike their sulfur analogs, selenium compounds are particularly effective at stoichiometric concentrations (Jacob, C.,

Maret, W., & Vallee, B. L. (1998) *Proc. Natl. Acad. Sci. USA* 95, 3489-3494). Therefore, we became aware of and interested in the selenium-containing drug ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), which mimics the catalytic action of glutathione peroxidase (Gpx), a property that its sulfur analog does not share (Müller, A., Cadenas, E., Graf, P., & Sies, H. (1984) *Biochem. Pharmac.* 33, 3235-3239). It is an excellent model for the action of selenocysteine, which owing to its instability cannot be studied itself. Ebselen reacts with thiols first by opening the isoselenazol ring while forming a sulfur-selenium bond. Excess thiols then reduce ebselen to a selenol derivative followed by the formation of a disulfide (Haenen, G. R. M. M., De Rooij, B. M., Vermeulen, N. P. E., & Bast A. (1990) *Mol. Pharmac.* 37, 412-422).

There is considerable interest in applications of this drug, because of its relatively broad therapeutic spectrum and its extremely low toxicity, which is being ascribed to the very poor bioavailability of selenium in ebselen (Sies, H. (1993) *Free Radical Biol. Med.* 14, 313-323; Schewe, T. (1995) *Gen. Pharmac.* 26, 1153-1169). It was explored with regard to its properties as an anti-inflammatory agent and as a general antioxidant. Ebselen inhibits a wide variety of enzymes, modifies the inflammatory response (Wendel, A., Fausel, M., Safayhi, H., Tiegs, G, & Otter, R. (1984) *Biochem. Pharmac.* 33, 3241-3245), and is now in phase III clinical trials in Japan to evaluate its effect on improving the outcome of ischemic stroke (Yamaguchi, T., Sano, K., Takakura, K., Saito, I., Shinohara, Y., Asano, T., & Yasahura, H. (1998) *Stroke* 29, 12-17).

As described in this example, ebselen reacts with MT and other biological zinc/sulfur coordination sites by concomitantly releasing zinc. This reaction is an example of the high reactivity and redox behavior of selenium toward thiols, and in particular illustrates the high reactivity which it retains even when the thiol is bound to zinc, thus demonstrating a completely new redox action of selenium, whose functional potential in selenocysteine has become appreciated only recently through the present work.

MATERIALS AND METHODS

Materials. Ebselen (98+ % pure) was obtained from Sigma (St. Louis, MO) and is of sufficiently low zinc content not to interfere with the zinc transfer assay. The extinction coefficients of ebselen in methanol (Sigma, sequencing grade) were determined as 14,400 $M^{-1}cm^{-1}$ (262 nm) and 6,000 $M^{-1}cm^{-1}$ (330 nm) (Morgenstern, R., Cotgreave, I. A., & Engman, L. (1992) *Chem.-Biol. Interactions* 84, 77-84). Rabbit MT-1 and MT-2 isoforms were converted from the cadmium-containing to the zinc-containing species (Vasak, M. (1991) *Meth. Enzymol.* 205, 41-44) and characterized by titration of thiols with 2,2'-dithiodipyridine and zinc analyses. Yeast alcohol dehydrogenase (Sigma) was subjected to

buffer exchange with the use of PD-10 gel filtration columns (Pharmacia). The enzyme contained 7.7 g-atoms of zinc per tetramer as determined by atomic absorption spectroscopy and spectrophotometric protein analyses (A_{280} 1% = 12.6), based on a molecular weight of 141,000. It was assayed spectrophotometrically with ethanol (0.1%) and NAD^+ (1 mM) in 0.1 M glycine/Na^+, pH 10.0 at 25 °C.

PAR (4-(2-pyridylazo)resorcinol) Zinc Transfer Assay. MT-2 (0.5 μM) and PAR (100 μM from a 1 mM stock solution in 20 mM Hepes, pH 7.5) were incubated with or without ebselen in 20 mM Hepes, pH 7.5 and both the rates and the total extent of zinc transfer calculated from the absorption of the formed zinc-PAR complex (ϵ_{500} = 65,000 $M^{-1}cm^{-1}$). Only the buffer was purged with nitrogen gas prior to use; hence, traces of dissolved oxygen remained during the assay.

UV-VIS Spectroscopy. Spectra were recorded with either Varian Cary 1 or Cary 50 spectrophotometers thermostatted at 25 °C. Stopped-flow kinetics were performed with a Biosequential SX-18MV reaction analyzer (Applied Photophysics, Leatherhead, U.K.). Selenol intermediates were assayed with 1-chloro-2,4-dinitrobenzene (CDNB) (Cotgreave, I. A., Morgenstern, R., Engman, L., & Ahokas, J. (1992) *Chem.-Biol. Interactions* 84, 69-76).

Reactivation of Apo-Carboxypeptidase A. Incorporation of zinc into the metal-depleted form of carboxypeptidase A (apo-CPA) was determined by spectrofluorimetric enzyme assays (Jacob et al., 1998) with a Spex Fluoromax-2 instrument.

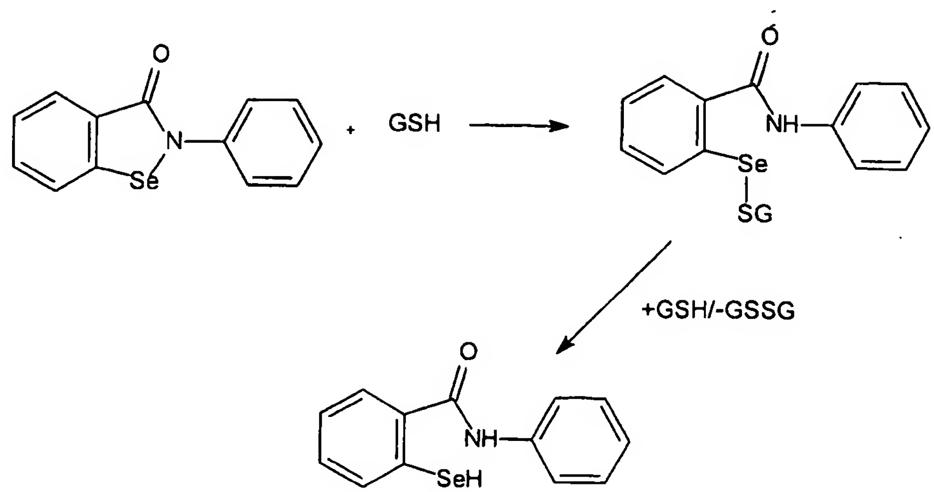
RESULTS AND DISCUSSION

Kinetics of the Reaction of Ebselen with Metallothionein. Ebselen at equimolar concentrations with respect to the twenty thiol ligands of MT affects a rapid ($t_{1/2}$ < 1 min) and complete transfer of zinc to either PAR (Fig. 14A) or apo-CPA (Fig. 14B) acting as zinc acceptors. The initial reactivation of carboxypeptidase A ("CPA") in the absence of ebselen is due to the residual activity of apo-CPA and zinc transfer from MT which can transfer up to one zinc atom (Jacob, C., Maret, W., & Vallee, B. L. (1998) *Proc. Natl. Acad. Sci. USA* 95, 3489-3494). Ebselen and its metabolites exhibit absorption spectra that are quite sensitive to chemical changes at the selenium atom (Ackerboom, T. P. M., Sies, H., & Ziegler, D. M. (1995) *Arch. Biochem. Biophys.* 316, 220-226). When the reaction between ebselen and MT is followed directly by wavelengths scans, two isosbestic points at 259 and 318 nm, characteristic of the interconversion of two absorbing species, are observed (Fig. 19A). They allow for the calculation of difference spectra which reveal changes below 250, at 282 ($\Delta\epsilon$ = 6,800 $M^{-1}cm^{-1}$), and at 340 nm $\Delta\epsilon$ = - 700 $M^{-1}cm^{-1}$ (Fig. 19B). One species is free ebselen whereas the other is a covalent adduct between ebselen and MT (selenenyl

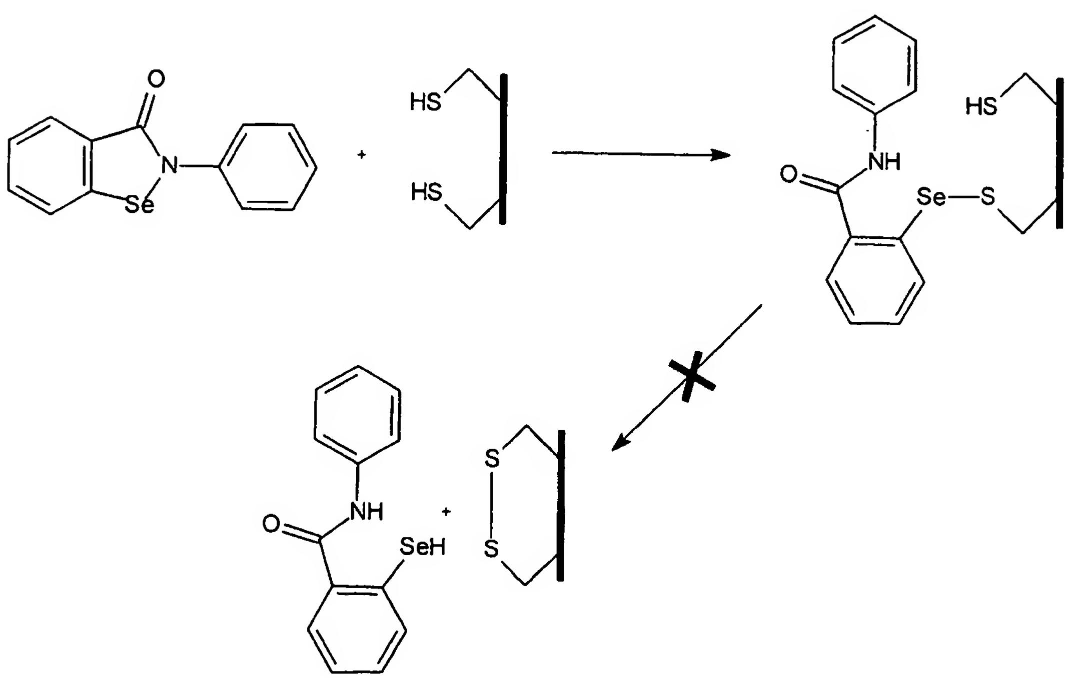
sulfide) formed after opening of the isoselenazonone ring (Wendel, A., Fausel, M., Safayhi, H., Tiegs, G, & Otter, R. (1984) *Biochem. Pharmac.* 33, 3241-3245). Therefore, the reaction of MT by ebselen can be studied at 282 nm. Kinetics followed at this wavelength in the absence of an acceptor are identical to those recorded with PAR, indicating that chemical modification of MT with ebselen rather than zinc release is the rate-limiting step. In order to gain more insight into the reactivity of the zinc/sulfur bonds with ebselen, a comparison with the reactivity of the thiol group in glutathione was performed under identical conditions. When followed at 282 nm, the reaction of ebselen with glutathione exhibits two kinetic phases instead of one, with difference extinction coefficients of 5,100 $M^{-1}cm^{-1}$ for the first phase and 8,200 $M^{-1}cm^{-1}$ for the entire reaction (data not shown). This is in agreement with an earlier study, in which the former phase was assigned to the formation of the seleneny1 sulfide (selenodisulfide with glutathione) and the latter to the formation of the diselenide (Haenen, G. R. M. M., De Rooij, B. M., Vermeulen, N. P. E., & Bast A. (1990) *Mol. Pharmac.* 37, 412-422). Because half-lives of these reactions could not be estimated accurately by this method, stopped-flow experiments were performed with 282 nm detection. The half-life of the reaction of ebselen with MT is about 5 sec (Fig. 20A) whereas that with glutathione is about 5 msec (Fig. 20B), reflecting the much faster reaction of a free thiolate in comparison with that of a zinc-bound thiolate. An excellent fit to a single, second order process, presumably reflecting the reaction of a single reactive cysteine in MT, was obtained.

When using CDNB as a reagent to assay for selenol (Cotgreave, I. A., Morgenstern, R., Engman, L., & Ahokas, J. (1992) *Chem.-Biol. Interactions* 84, 69-76), a reactive selenol intermediate was detected only in the reaction of ebselen with glutathione. Therefore, this reaction proceeds to the selenol (-SeH) (Scheme A), in contrast to the reaction of ebselen with MT which stops at the step of the covalent adduct (Scheme B). Inter- or intramolecular attack of the selenium-sulfur bond in the MT-ebselen adduct by a thiolate would be required if the reaction were to proceed further. In MT, this seems to be either sterically hindered or the remaining bound zinc decreases dramatically such a reactivity. Cleavage of the adduct is achieved, however, when glutathione is added in excess after the reaction with MT and substoichiometric amounts of ebselen has taken place. In this case, the selenol derivative is detected with CDNB . In conclusion, there are qualitative as well as quantitative differences between the reactions of ebselen with glutathione on one hand and that of ebselen with MT on the other: Zinc/thiolate bonds react significantly slower than free thiolates and a selenol intermediate is not formed in the reaction with MT.

Scheme A



Scheme B



When a titration of MT with ebselen is performed in which zinc release to PAR is followed to completion after addition of each aliquot of ebselen, there is a linear relationship between zinc released and ebselen added until a plateau is reached at 20 ebselen

molecules per MT molecule (Fig. 21). Thus ebselen reacts stoichiometrically with all 20 cysteines in MT.

Reaction of Ebselen with other Zinc/Sulfur Centers. In order to explore whether or not the reaction of ebselen is unique for the clusters in MT, we have investigated its reaction with yeast alcohol dehydrogenase. This enzyme binds two zinc ions per subunit, the first a catalytic zinc with one histidine and two cysteine ligands and the second a structural zinc with four cysteine ligands. Twenty ebselen molecules are required to release all of the zinc atoms from this enzyme (Fig. 22), close to the expected value of 24 (six cysteine ligands in each of the four subunits). At the same time, alcohol dehydrogenase activity decreases to less then 20% of its original value, suggesting a stoichiometric reaction of ebselen with cysteine in the structural and catalytic sites of the enzyme. Alternatively, the two additional cysteines in the enzyme (Jörnvall, H. (1977) *Eur. J. Biochem.* 72, 443-452) might react and zinc is ejected when less than all six cysteine ligands are modified. The reaction of ebselen with alcohol dehydrogenase is significantly slower (half-life about 5-10 min) than that with MT (data not shown). Thus, the reactivity of MT is between that of free thiols and that of a complex in which all thiolates are bound as terminal ligands, suggesting that the presence of bridging thiolate ligands in the clusters increase the reactivity MT. This also suggests that ebselen may react preferrentially with MT *in vivo*.

Reaction of Ebselen with MT in the Presence of Glutathione. Ebselen reacts efficiently with thiols such as glutathione to form a selenodisulfide (Sies, H. (1993) *Free Radical Biol. Med.* 14, 313-323). Therefore, MT was incubated with ebselen in the presence of glutathione as a competitor. Even in the presence of a 4-fold excess of glutathione (2-min pre-incubation) zinc was released from MT, albeit at a lower rate (data not shown). Since glutathione reacts much faster with ebselen than with MT (see above), we conclude that in this experiment ebselen selenodisulfide instead of ebselen is the species that reacts with MT. This result, therefore, provides a mechanism of how ebselen could react with MT *in vivo* despite the relatively high competing concentrations of glutathione (0.1 -10 mM), an important implication. *In vivo,* there could also be redox-cycling of the glutathione ebselen adduct (selenodisulfide) and the diselenide which re-generate ebselen in the presence of hydrogen peroxide (Sies, H. (1993) *Free Radical Biol. Med.* 14, 313-323), a reaction sequence that closes a catalytic cycle. Furthermore, ebselen metabolites are oxidized enzymatically to compounds that also act as thiol oxidants (Ackerboom, T. P. M., Sies, H., & Ziegler, D. M. (1995) *Arch. Biochem. Biophys.* 316, 220-226). The existence of multiple pathways leading to metabolites of ebselen that oxidize thiols strongly suggests that zinc release is part of the therapeutic spectrum of ebselen.

Mechanistic Considerations. Overall, the data provide the first evidence that zinc-bound thiolates are a target for ebselen. Selenium in the isoselenazole (Se-N bond) ring of ebselen reacts not only with free thiols, either in the form of glutathione or enzyme active sites such as glutathione S-transferase or papain (Nikawa, T., Schuch, G., Wagner, G., & Sies, H. (1994) *Biochem. Pharmac.* 47, 1007-1012) but also with thiols when bond to zinc as in MT. In this case, ebselen forms a rather stable adduct and the consecutive reaction, the formation of a disulfide bond in MT and the concomitant release of a reduced selenol derivative of ebselen, seems to be rather slow if it occurs at all. Ebselen shows little catalytic activity toward the hydrogen peroxide-induced zinc release from MT-2, also indicating the absence of a selenol intermediate. Thus, the reaction of ebselen with MT is not catalytic and ebselen does not act as a glutathione peroxidase mimic in this reaction. With other thiols, ebselen reacts *reversibly* in the presence of hydrogen peroxide and, hence will be available for the *irreversible* reaction with MT in the presence of glutathione.

Implications. The action of ebselen further supports the new principle that MT and oxidizing agents react with concomitant zinc release (Maret, W. & Vallee, B. L. (1998) *Proc. Natl. Acad. Sci. USA* 95, 3478-3482). The stoichiometric reaction of ebselen with MT and ensuing mobilization of zinc is evidence for a hitherto unknown relation between zinc and selenium. In particular, since the reaction is one of the fastest observed so far, it points at possible reactions that might be carried out *in vivo* by selenocysteine, the only known selenium-containing functional amino acid in proteins. Selenium is an important antioxidant (Bettger, W. J. (1993) *Can. J. Physiol. Pharmacol.* 71, 721-724) as is ebselen whose properties as a glutathione peroxidase mimic involve both reduction of peroxides and oxidation of thiols. The mechanism of action of ebselen as a therapeutic drug is not understood fully. In the reaction with MT, ebselen oxidizes thiols in MT and, therefore, actually acts as an oxidant. Controlled release of zinc, which itself is also considered an antioxidant (Bray, T. M. & Bettger, W. J. (1990) *Free Radical Biol. Med.* 8, 281-291), could contribute to the *overall* antioxidant action of ebselen. This is a new mechanism of action of this drug, which might underlie its known actions and certainly provides an opportunity for further exploration of its possible antiviral effects by acting on viral zinc finger proteins, other antioxidant actions of zinc, or specific release of zinc as an activator of apoenzymes.

6.4.2. <u>CATALYTIC ACTIVITY OF THE NATURALLY OCCURING AMINO ACID SELENOCYSTINE IN ZINC RELEASE FROM METALLOTHIONEIN</u>

Selenocysteine is a natural occurring amino acid which forms the active center of a number of enzymes, notably glutathione peroxidase and thyroid deiodinase. While the reaction of diselenides with glutathione has been studied previously, virtually nothing is known about the interactions of diselenides with zinc-sulfur bonds. Therefore oxidative zinc release from MT was studied in the presence of D,L-selenocystine. This reagent releases zinc from MT in the absence of an additional oxidant. However, they were also found to enhance zinc transfer from MT to PAR (4-(2-pyridylazo)resorcinol) when ^{t}butylhydroperoxide (^{t}BuOOH) was used as mild oxidizing agent.

MATERIALS AND METHODS

Materials. D,L-selenocystine and ^{t}BuOOH were obtained from Sigma (St. Louis, MO) and are of sufficiently low zinc content not to interfere with the zinc transfer assay. Rabbit MT-1 and MT-2 isoforms were converted from the cadmium-containing to the zinc-containing species (Vasak, M. (1991) Meth. Enzymol. 205, 41-44) and characterized by titration of thiols with 2,2'-dithiodipyridine and zinc analysis.

PAR Zinc Transfer Assay. MT (0.5 μM) and PAR (100 μM from a 1 mM stock solution in 20 mM Hepes, pH 7.5) were incubated with or without D,L-selenocystine and ^{t}BuOOH in nitrogen purged 20 mM Hepes, pH 7.5 and both rates and the total extent of zinc transfer calculated from the absorption of the formed zinc-PAR complex ($\epsilon_{500} = 65{,}000\ M^{-1}cm^{-1}$). Although the buffer was purged with nitrogen gas prior to use, traces of dissolved oxygen remained during the assay.

UV-VIS Spectroscopy. Spectra were recorded with a Varian Cary 1 spectrophotometer thermostatted at 25°C.

RESULTS AND DISCUSSION

Kinetics of the reaction of D,L-Selenocystine with Metallothionein. D,L-selenocystine at equimolar concentrations with respect to the twenty thiol ligands of MT affects transfer of zinc to PAR. Interestingly, zinc transfer under those conditions cannot be quenched by even a 30 fold excess of glutathione. This observation might indicate some specificity of D,L-selenocystine (or intermediates) towards MT in comparison to glutathione. Additionally, as can be seen in Figure 23, substoichiometric amounts of D,L-selenocystine (50 nM compared to 10 μM thiols) enhance zinc transfer in the presence

of ^tBuOOH. This indicates that this biological selenium compound acts catalytically on the reaction of MT with a peroxide resulting in zinc release. Diselenides are reducible by thiols to selenols. As a consequence, a catalytic cycle involving selenol and selenenic acid seems to be most likely. These intermediates form part of the glutathione peroxidase redox cycle.

6.5. BINDING OF ATP TO METALLOTHIONEIN

6.5.1. ATP AND ZINC RELEASE FROM METALLOTHIONEIN

Abstract. ATP binds to MT (K_D = 176 +/- 33 μM at pH 7.4), enhances transfer of zinc to an acceptor such as zinc-depleted sorbitol dehydrogenase, and increases the rate of thiol/disulfide interchange with Ellman's reagent about twofold. GTP has almost identical effects. The corresponding di- or monophosphates and pyrimidine nucleotides, however, neither bind as strongly as ATP nor do they enhance zinc transfer. Carbamylation of lysines of MT abolishes the binding of ATP, indicating that the highly conserved lysines are part of the binding site. The binding of ATP is affected by glutathione and glutathione disulfide, both of which modulate zinc release and its transfer from MT (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488). Glutathione decreases ATP binding whereas glutathione disulfide increases it.

Introduction

The two metal clusters in metallothionein (MT) are formed by seven zinc atoms bound to twenty sulfur atoms ($K_d = 1.4 \times 10^{-13}$ M at pH 7.0 for human MT [Kagi et al., 1993]) and constitute the reservoir for storage, release, and transfer of zinc from and to other proteins (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488). The thermodynamic stabilities and kinetic labilities of zinc in MT are the consequence of ligand exchange and redox reactions of the cysteine sulfur donor atoms (Maret, W., Larsen, K. S., & Vallee, B. L. (1997) Proc. Natl. Acad. Sci. USA, 94, 2233-2237; Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3478-3482). We have shown further that the interactions of MT with glutathione and glutathione disulfide or other oxidizing agents control the state of zinc in MT, demonstrating that the zinc content of MT is a function of the cellular redox state. Phosphate, the only other known biological MT ligand, is required for the formation of stable MT crystals (Robbins, A. H., McRee, D. E., Williamson, M., Collett, S. A., Xuong, N. H., Furey, W. F., Wang, B. C., & Stout, C. D. (1991) J. Mol. Biol., 221, 1269-1293), and binds to the carbonyl group of Cys-19 and to the ε-amino group of Lys-31 which places it between the two domains and acts as linker

because it engages in the only interdomain hydrogen bonds with the carbonyls of Cys-19 and Cys-21 (Robbins, A. H., McRee, D. E., Williamson, M., Collett, S. A. , Xuong, N. H., Furey, W. F., Wang, B. C., & Stout, C. D. (1991) J. Mol. Biol., 221, 1269-1293). It is also known to bind with relatively high affinity to dimeric Cd-MT (Palumaa, P., Zerbe, O., & Vasak, M. (1993) Biochemistry, 32, 2874-2879) or to monomeric Cd-MT in the presence of excess cadmium (Palumaa, P., Zerbe, O., & Vasak, M. (1993) Biochemistry, 32, 2874-2879). The physiological significance of phosphate binding is not known, however.

We have continued our search for biological ligands that interact with MT. The facts that GTP binds to MT (cited in Vallee, B. L. (1979) Experientia Suppl., 34, 19-40) and that the Zn-ATP complex is a substrate for pyridoxal kinase, which is activated by Zn-MT (Churchich, J. E., Scholz, G., & Kwok, F. (1989) Biochim. Biophys. Acta, 996, 181-186) led us to investigate the binding of nucleotides to MT and its possible effects on the state of zinc, its transfer to and from MT, and the resultant reactivities of this molecule. We have now established that both ATP and GTP bind to MT and that the binding may be limited to purine nucleotides.

Materials and Methods

Materials. Nucleotides, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and rabbit liver Cd,Zn-metallothionein II (Cd,Zn-MT-2) were obtained from Sigma; $^{65}ZnCl_2$ (77.7-103.6 Gbq/g) from Dupont NEN; sheep liver sorbitol dehydrogenase (SDH) from Boehringer Mannheim; adenosine 5'-[β,γ-imido]triphosphate (AMP-PNP) from Fluka; and 1,N^6-ethenoadenosine 5'-triphosphate (ϵ-ATP) from Molecular Probes. Zinc-depleted sorbitol dehydrogenase (apo-SDH) and human Zn_7-MT-2 were prepared and characterized as described (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488).

Lysine Modification. Lysines in MT were modified by dissolving rabbit liver Cd,Zn-MT-2 in 0.2 ml of saturated sodium borate, pH 9.2, adding solid potassium cyanate to a final concentration of 1 M and incubating the reaction mixture for 40 h at 37 °C (Zeng, J. (1991) Meth. Enzymol., 205, 433-437). Excess salt was removed by at least 6 dilution/concentration cycles using Centricon 3 centrifugal microconcentrators (Amicon).

Zinc transfer from MT to apo-SDH in the presence of nucleotides. Apo-SDH (1.7 μM) was incubated with Zn_7-MT (0.24 μM) and the nucleotide (1 mM) to be tested in 0.2 M Tris-HCl, pH 7.4 at 22.5±0.5°C. Aliquots (10 μl) were withdrawn periodically and assayed for enzymatic activity (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488).

Reaction of ^{65}Zn-MT-2 with ATP. Human $^{65}Zn_7$-MT-2 (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488) (0.76 μM) was incubated with 1 mM ATP at 22.5±0.5 °C for one hour. The reaction mixture was then separated on a DEAE MemSep-1000 chromatography cartridge (Millipore) using a linear, 10-min gradient from 0 to 75 mM sodium chloride in 10 mM Tris-HCl, pH 8.6 at a flow rate of 5 ml/min. Radioactivity in each fraction was measured by γ-emission spectroscopy with a Searle model 1185 Automatic Gamma system operating at a 0.12-1.2 MeV energy range.

Reactivity of MT in the presence of nucleotides. The thiol reactivity of MT was determined using DTNB under pseudo-first order rate conditions where [DTNB]<[MT] (Cismowski, M. J., & Huang, P. C. (1991) Biochemistry, 30, 6626-6632). Under these conditions two equivalents of thiobenzoate are formed, since the inter- or intramolecular disulfide of MT is favored over the mixed disulfide (Savas, M. M., Shaw, C. F. III, & Petering, D. H. (1993) J. Inorg. Biochem., 52, 235-249). The reaction between MT (10 μM) and DTNB (4 μM) without or with nucleotide (1 mM) in 0.2 M Tris, pH 7.4 was followed spectrophotometrically at 412 nm and 25 °C.

ATP binding. a) Electronic absorption difference spectra. Double difference spectra of Zn-MT (2.5 μM) with different concentrations of ATP were recorded in 0.2 M Tris, pH 7.4 on a Cary model 1E high performance UV-Vis spectrophotometer (Varian) using tandem cuvets (Roustan, C., Kassab, R., Pradel, L. A., & Thoai, N. V. (1968) Biochim. Biophys. Acta, 167, 326-338). ATP and MT were separated in the two chambers of the reference cuvet, while they were mixed in the chambers of the sample cuvet. Spectra were recorded (spectrum 1), the samples in the two chambers of the reference cuvet were mixed, and the spectra were recorded again (spectrum 2). Double difference spectra were obtained by subtracting spectrum 2 from spectrum 1.

b) Hummel-Dreyer method (Hummel, J. P., & Dreyer, W. J. (1962) Biochim. Biophys. Acta, 63, 530-532.) A Sephadex G-25 column (1 x 30 cm) was equilibrated with 50 mM Hepes buffer, pH 7.4 containing ATP (100 μM). Cd,Zn-MT was dissolved in this buffer and then applied to the column. If MT binds to ATP, the resulting elution profile will exhibit a peak at the position where MT elutes, and a trough, representing the depletion of ATP in the buffer, at the position where ATP would elute. From the area of the trough and the known amount of protein applied, the binding ratio was determined at each concentration of ATP.

c) Fluorescence quenching. The fluorescent ATP analogue ϵ-ATP was dissolved in 0.2 M Tris-HCl, pH 7.4. Emission spectra were recorded with a FluoroMax-2 spectrofluorimeter (Instruments S.A., Inc.) at an excitation wavelength of 274 nm with 5 nm slit widths.

Results

Zinc transfer from MT to apo-SDH in the presence of nucleotides.: In the absence of any other agents, the reconstitution of apo-SDH with Zn_7-MT-2 is slow but reaches 13% after one hour of incubation at equimolar concentrations of apo-SDH and zinc in MT (Fig. 24). Full activation requires equimolar concentrations of apo-SDH and MT and under these conditions only one of the seven zinc atoms of MT is transferred ($k_2 = 16\ M^{-1}s^{-1}$) (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488). ATP (1 mM) increases the rate of the reaction about 18-fold ($k_2 = 288\ M^{-1}s^{-1}$) and the extent almost 6-fold, leading to 74% reactivation (Fig. 24). The comparison of rate constants is based on the assumption that in both cases only one zinc atom is transferred. In fact, however, in the presence of ATP at least five zinc atoms are released from MT and transferred to apo-SDH. The enhancement of zinc transfer depends on the concentration of ATP; 10 μM ATP leads to 21% and 100 μM to 36% reactivation, respectively (data not shown). GTP (1 mM) similarly increases the rate of zinc transfer 13-fold ($k_2 = 207\ M^{-1}s^{-1}$) and the number of zinc atoms transferred to four. These effects are of the same order of magnitude as those observed with the glutathione/glutathione disulfide system (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488). However, other purine nucleotides, e.g. ADP (Fig. 24), AMP, GMP, *c*AMP and *c*GMP or pyrimidine nucleotides such as CTP or UTP, do not enhance zinc transfer from MT at all. ATP often binds to other biomolecules in the form of a Mg or Ca complex. Hence, these possibilities were examined. However, neither calcium nor magnesium ions at concentrations of up to 1 mM affected the reaction in any way. ATP analogs such as ATP-γ-S and AMP-PNP are hydrolyzed poorly and therefore are commonly employed to examine whether or not ATP hydrolysis is a component or accompaniment of a physiological process. The reactivation of apo-SDH by MT in the presence of AMP-PNP closely resembles that in the presence of ATP, indicating that ATP hydrolysis is not essential for zinc transfer from MT (Fig. 24). Vanadate, a potent ATPase inhibitor does not affect zinc transfer.

ATP is a relatively weak zinc-chelating reagent ($\log K = 5.2$) with a binding constant for zinc close to that of zincon ($\log K = 4.9$). To test whether or not the effect of ATP solely relates to its capacity to sequester zinc, we have examined the effect of zincon on the zinc transfer assay. Zincon fails to enhance zinc transfer from MT to apo-SDH. Three types of experiments support the conclusion that ATP by itself does not release zinc from MT. When 4-(2-pyridylazo)resorcinol (PAR) serves as a zinc acceptor, ATP (1 mM) does not release zinc from MT. ATP also does not release zinc from ^{65}Zn-labelled MT as demonstrated by anion exchange chromatography. Zinc measurements of fractions from

Hummel and Dryer chromatography show that at most 7% of zinc is released from MT. Hence the mode of action of ATP in zinc transfer to and from MT cannot be compared to that of a chelating agent. Moreover phosphate or polyphosphate fails to enhance zinc transfer to apo-SDH.

Binding of ATP to MT. If the binding of a chromophoric ligand to a protein changes the latter's intrinsic spectroscopic properties, difference spectra can become a powerful method to detect and characterize binding. Double difference spectra of ATP binding to MT at different concentrations of ATP (Fig. 25) demonstrate that ATP binds to MT. Half-maximal saturation occurs at about 140 μM, but there is no significant wavelength shift compared to free ATP ($\Delta\epsilon$ = 14,400 $M^{-1}cm^{-1}$).] ADP and AMP (100 μM) elicited less than 10% of the effect of ATP at an identical concentration.

Use of the Hummel-Dreyer method confirmed this relatively weak binding of ATP to MT (Fig. 26). The stoichiometry and binding constant were obtained from the Scatchard equation (Segel, I. H. (1976) Biochemical Calculations (John Wiley, New York), pp 241-244).

$$[ATP_B]/ [ATP_F][MT]_0 = -[ATP_B] / K_d[MT]_0 + n / K_d$$

where $[ATP_B]$ and $[ATP_F]$ are the concentrations of bound and free ATP, respectively; $[MT]_0$ the initial concentration of MT, n the number of identical and independent binding sites of MT, and K_d the dissociation constant. The linear Scatchard plot gave a value of 0.93 for n (Fig. 27) - indicating that MT binds one molecule of ATP with a K_d of 176 (+/- 33) μM, close to the value estimated by double difference spectrophotometry (Fig. 25). A similar elution profile exhibiting a peak and a trough was observed for GTP, but not for UTP. In contrast, slight changes observed with ADP are within the experimental error. Thus, the preferential binding seems to be limited to purine nucleotides which are also the ones that affect zinc transfer from MT (see above).

Eight lysine residues are relatively highly conserved in mammalian MT-1/-2 isoforms. Since they might participate in ATP binding, they were modified by carbamoylation (Zeng, J. (1991) Meth. Enzymol., 205, 433-437). ATP binding to lysine-modified MT is essentially abolished as assayed by the Hummel-Dreyer method. Further, ATP does not enhance zinc transfer of lysine-modified MT to apo-SDH, demonstrating that ATP binding is a prerequisite for the enhancement of zinc transfer. Since phosphate binds to Lys-31 (Robbins, A. H., McRee, D. E., Williamson, M., Collett, S. A. , Xuong, N. H., Furey, W. F., Wang, B. C., & Stout, C. D. (1991) J. Mol. Biol., 221, 1269-1293), we have investigated the binding of ATP in the presence of 50 mM phosphate. This relatively large

excess of phosphate significantly suppresses the ATP binding, indicating that phosphate and ATP compete for a binding site that involves Lys-31.

Binding of a fluorescent ATP analogue. ϵ-ATP, a fluorescent analogue of ATP, was used as an alternative method to visualize ATP binding to MT (Fig. 28). When it binds to MT quenching of fluorescence is observed (Fig. 28). The magnitude of the quenching is likely due to the cadmium in MT, since a significantly smaller quenching is observed when Zn-MT was used in an analogous experiment. Half-maximal quenching occurs at 20 μM of ϵ-ATP, which is about one seventh of the K_d value observed for ATP. Binding of the analogue, therefore, is significantly stronger as has been observed in other instances where this was explained on the basis of the additional hydrophobic surface in comparison with ATP (Sigel, H. and Song, B. (1996) in Interactions of Metal Ions with Nucleotides, Nucleic Acids, and Their Constituents eds., Sigel, A. & Sigel, H., Metal Ions in Biological Systems, 32, 135-205, Marcel Dekker, New York).

ATP binding in the presence of glutathione. It has been suggested that the zinc content of MT is controlled by the cellular glutathione redox state (Maret, W. (1994) Proc. Natl. Acad. Sci. USA 91, 237-241; Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488). Hence, the effect of GSH and GSSG on the binding of ATP to MT was examined. GSH inhibits the binding of ATP to MT whereas GSSG enhanced the binding (Fig. 29).

Sulfhydryl Reactivity of MT in the presence of nucleotides. The reactivity of the sulfhydryl groups of MT is one of the most sensitive probes of the state of the conformation of MT. In order to test whether or not nucleotide binding has any effect on the reactivity of the molecule we investigated the reaction of MT with DTNB. ATP (1 mM) increased the rate of the reaction significantly (Fig. 20A). Standard kinetic treatment of the data demonstrates that the reaction can be described by a single process with a rate constant of $2.8 +-0.3 \times 10^{-3} s^{-1}$ in the absence of ATP and of $5.3 +-0.4 \times 10^{-3} s^{-1}$ in its presence (Fig. 30B). Similarly GTP increased the reactivity 2.2-fold, while AMP had no effect.

Discussion

MT does not have a consensus ATP or GTP binding motif (Kjeldgaard, M., Nyborg, J., & Clark, B. F. C. (1996) FASEB J., 10, 1347-1368). In contrast to other systems with ATP/GTP recognition sites where ATP binds in the micromolar range and GTP binds in the nanomolar range, the binding of ATP to MT is relatively weak and out of the range of conventional assays for tight binding (Jakob, U., Scheibel, T., Bose, S., Reinstein, J., & Buchner, J. (1996) J. Biol. Chem., 271, 10035-10041). For example, binding of phosphate

to MT dimers can be detected using a gel filtration assay (Palumaa, P. & Vasak, M. (1992) Eur. J. Biochem., 205, 1131-1135), since the dissociation constant is 14 μM (pH 7.4). However, this method is not suitable for measuring ATP binding to MT because the MT-ATP complex will dissociate on the column. Therefore, an equilibrium method such as the one proposed by Hummel and Dreyer (Hummel, J. P., & Dreyer, W. J. (1962) Biochim. Biophys. Acta, 63, 530-532) has been employed here to determine the stability constant of the MT-ATP complex. Though weak, the binding is specific for purine nucleotide triphosphates, since binding of other nucleotides could not be detected. Also, the binding data correlate well with the data for zinc transfer from MT to apo-SDH. It is perhaps of significance that only the purine nucleotides show these effects. They are known to form more stable complexes with zinc due to the formation of a macrochelate involving N-7 of the base (Sigel, H. and Song, B. (1996) in Interactions of Metal Ions with Nucleotides, Nucleic Acids, and Their Constituents eds., Sigel, A. & Sigel, H., Metal Ions in Biological Systems, 32, 135-205, Marcel Dekker, New York). At least in two cases, interaction of N-7 of ATP with a zinc atom in an enzyme has been demonstrated (Wu, F.Y.-H., Huang, W.-J., Sinclair, R. B., & Powers, L. (1992) J. Biol. Chem., 267, 25560-25567; Wilson, D. K., & Quiocho, F. A. (1993) Biochemistry, 32, 1689-1694). It is quite possible that purine nucleotides have been chosen as ligands, because they form complexes that could act as shuttles of zinc. This function would require that the nucleotide is not bound very strongly.

The total amount of cellular GTP is about 0.3 - 0.6 mM (Hatakeyama, K., Harada, T., & Kagamiyama, H. (1992) J. Biol. Chem., 267, 20734-20739), roughly one order of magnitude lower than that of ATP (see below). Hence, MT should form preferentially a complex with ATP. We are, therefore, restricting our discussion here to this nucleotide.

Experiments with lysine-modified MT indicate that lysines are involved in the binding of ATP, most certainly Lys-31 as this residue binds orthophosphate in the crystal structure of MT (Robbins, A. H., McRee, D. E., Williamson, M., Collett, S. A. , Xuong, N. H., Furey, W. F., Wang, B. C., & Stout, C. D. (1991) J. Mol. Biol., 221, 1269-1293). At least eight lysines are conserved in the protein. They were thought to be necessary for stabilizing the negatively charged clusters (Pande, J., Vasak, M., & Kagi, J. H. R. (1985) Biochemistry, 24, 6717-6722). Lysine-modified MT apparently binds all seven zinc ions (Zeng, J. (1991) Meth. Enzymol., 205, 433-437). In addition, mutagenesis experiments, in which all lysines in the alpha-domain were replaced by glutamates did not show that the lysines are critical for the structure of the protein (Pan, P. K., Hou, F. Y., Cody, C. W., & Huang, P. C. (1994) Biochem. Biophys. Res. Commun., 202, 621-628). Thus, it is not clear what role the lysines have. Our results suggest that the lysines are conserved because they

take part in nucleotide binding. The lysines could also be part of a nuclear translocation sequence. Nuclear translocation of MT requires energy and the "nucleophilic" distribution of MT in human tumor cells is related to the ATP state (Woo, E. S., Kondo, Y., Watkins, S. C., Hoyt, D. G., & Lazo, J. S. (1996) Experimental Cell Research, 224, 365-371).

Given the relatively low affinity of ATP for MT, the question arises whether or not MT is associated with ATP in the cell. If one takes into account that the cytosolic concentration of ATP is under tight control and is almost always in the millimolar range (Peters, G. J., De Arbreu, R. A., Oosterhof, A., & Veerkamp, J. H. (1983) Biochim. Biophys. Acta, 759, 7-15; Rauch, U., Schulze, K., Witzenbichler, B., & Schultheiss, H. P. (1994) Circulation Res., 75, 760) while MT is present in the micromolar range or below (Krezoski, S. K., Villalobos, J., Shaw III, C. F., & Petering, D. H. (1998) Biochem. J., 255, 483-491), one can calculate that MT is essentially saturated with ATP in the cell. We have discussed previously that given the typical cellular concentrations of glutathione, MT must be saturated with glutathione as well (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488). Glutathione concentrations seem to change over a much wider range than those of ATP (Meister, A., J. Biol. Chem, 262, 17205-17208). Since glutathione inhibits the binding of ATP (Fig. 28) and the binding of ATP is related to an enhancement of zinc transfer, these data reinforce our conclusion that the concentration of glutathione is an important modulator of zinc transfer reactions of MT (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488). Since at least two ligands of MT, ATP and glutathione, bind *in vivo*, isolated MT is always devoid of these "cofactors". It may be that in the absence of these cofactors MT adopts a conformation where the zinc atoms are almost completely shielded from the environment. As our experiments show, neither cofactor releases zinc but each activate the protein for reactions with oxidants such as disulfides.

As discussed below, without committing ourselves to any particular mechanism, ATP could change the conformation of MT (maybe the small binding energy is sufficient for inducing a large conformational change in a rather flexible small protein such as MT), thereby influencing the reactivity of the zinc clusters. The observation that zinc transfer is observed only in the presence of apo-SDH indicates that ATP "loosens the conformation" for more efficient zinc transfer. We have found that the binding of ATP per se does not release zinc, but that it indeed increases the reactivity of thiols toward disulfides. Thus ATP modulates the redox behavior of MT in a manner that is analogous to that of GSH. GSH has been proposed to bind to the β-domain of MT by displacing a thiol ligand (Brouwer, M. Brouwer, T. H., & Cashon, R. E. (1993) Biochem. J., 294, 219-225). We

have proposed that this free thiol is more reactive toward disulfides such as GSSG (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. USA, 95, 3483-3488). Here, we used Ellmans' reagent (DTNB) to investigate the redox behavior of MT. This reaction has been studied in great detail (Li, T.-Y., Minkel, D. T., Shaw, C.F. III, & Petering, D. H. (1981) Biochem. J., 193, 441-446; Cismowski, M. J., & Huang, P. C. (1991) Biochemistry, 30, 6626-6632; Savas, M. M., Shaw, C. F. III, & Petering, D. H. (1993) J. Inorg. Biochem., 52, 235-249).

6.5.2. ATP AND THE CONFORMATION OF METALLOTHIONEIN

The following experiment illustrates a relatively significant change in the shape of MT when ATP is bound.

Our previous work has shown that ATP binds to metallothionein (MT) and that this binding increases both the reactivity of MT towards disulfides and the transfer of zinc from MT to the apoform of sorbitol dehydrogenase. These observations suggest that ATP affects the conformation of MT. To test this hypothesis we performed gel filtration experiments of MT in the presence and absence of ATP.

Materials:

ATP (A-7699), Cd,Zn-MT-2 (M-5392) and dithiodipyridine (D-5767) were obtained from Sigma; Sephadex G-75 from Pharmacia.

Methods:

A Sephadex G-75 column (1 x 120 cm) was equilibrated with 50 mM Na^+/Hepes (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), pH 7.4 containing 10 mM NaCl without (for the experiment with MT) and with 1 mM ATP. MT (0.5 mg) was dissolved in 200 μL of this buffer with or without ATP, loaded onto the column and eluted at a flow rate of 6.7 mL/h. Because of the high absorbance of the nucleotide in the ultraviolet, MT was located by spectrophotometric determination of the sulfhydryl groups with dithiodipyridine (ϵ_{343} = 7600 $M^{-1}cm^{-1}$).

Results:

As shown in Figure 31, the MT/ATP complex elutes about 20 min later than MT (Figure). It was already known that MT itself, owing to the prolate ellipsoid shape of the molecule,

elutes from gel filtration columns at a position that corresponds to about twice its molecular weight.

Without committing ourselves to any particular mechanism, the large shift of the MT peak upon binding of ATP in our experiment suggests that the shape of the molecule changes to that of a globular protein. In a pictorial presentation of the conformational change we envision a venus-fly trap model, in which the ATP molecule becomes sandwiched between the two domains of the MT molecule.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications, as would be obvious to a person skilled in the art, are intended to be included in the scope of the following claims.

WE CLAIM:

1. A method for modulating the release of zinc from metallothionein within a cell comprising altering the concentrations of glutathione and glutathione disulfide within the cell.

2. A method of promoting the release of zinc from metallothionein within a cell comprising increasing the concentration of glutathione disulfide within the cell.

3. A method of promoting the release of zinc from metallothionein within a cell comprising increasing the concentration of glutathione disulfide within the cell in the presence of naturally occurring glutathione.

4. A method of promoting the release of zinc from metallothionein within a cell comprising administering to the cell a zinc-releasing amount of an oxidizing agent.

5. The method according to claim 4 wherein said oxidizing agent is selected from the group consisting of ebselen, selenocystine, selenocystamine, selenoglutathione disulfide, iron(III) chelates, dehydroascorbate, glutathione disulfide, coenzyme A disulfide, cystamine, FAD, NAD^+, copper (II) chelates, and combinations thereof.

6. The method according to claim 4 wherein said oxidizing agent is selected from the group consisting of ebselen, glutathione disulfide, and combinations thereof.

7. The method according to claim 4 wherein said oxidizing agent is ebselen.

8. A method of inhibiting the release of zinc from metallothionein within a cell comprising increasing the concentration of glutathione within the cell.

9. A method of inhibiting the release of zinc from metallothionein within a cell comprising increasing the concentration of glutathione within the cell in the presence of naturally occurring glutathione disulfide.

10. A method of inhibiting the release of zinc from metallothionein within a cell comprising administering to the cell an amount of a reducing agent effective to inhibit the release of zinc from metallothionein.

11. The method according to claim 10 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

12. The method according to claim 10 wherein said reducing agent is selected from the group consisting of glutathione, ascorbate, vitamin E, and combinations thereof.

13. The method according to claim 1 wherein said cell is a cultured cell.

14. The method according to claim 1 wherein said cell is contained in a host organism.

15. The method according to claim 14 wherein said organism is a plant.

16. The method according to claim 14 wherein said organism is an animal.

17. The method according to claim 16 wherein said animal is a human.

18. A method of treating a subject having a pathological condition in which zinc homeostasis is perturbed, comprising administering to the subject an amount of a compound effective to treat said condition, wherein said compound changes the cellular oxidation state to effect an action selected from the group consisting of causing a release of zinc from metallothionein and preventing release of zinc from metallothionein.

19. The method according to claim 18 wherein said compound is selected from the group consisting of ebselen, cytochrome c, ascorbate, dehydroascorbate, glutathione, glutathione disulfide, coenzyme A, cystamine, cysteamine, FAD, $FADH_2$, azurin, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

20. The method according to claim 18 wherein said compound is ebselen, ascorbate, dehydroascorbate, glutathione, glutathione disulfide, cystamine, cysteamine, azurin, vitamin E, N-acetyl-L-cysteine, or α-lipoate.

21. The method according to claim 18 wherein said compound is ebselen.

22. The method according to claim 18 wherein said pathological condition is a disease of the central nervous system, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the disease of the central nervous system.

23. The method according to claim 22 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

24. The method according to claim 18 wherein said pathological condition is Alzheimer's disease, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the Alzheimer's disease.

25. The method according to claim 24 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

26. The method according to claim 18 wherein said pathological condition is a neurodegenerative disease, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the neurodegenerative disease.

27. The method according to claim 26 wherein said neurodegenerative disease is selected from the group consisting of Parkinson's disease, and amyotrophic lateral sclerosis.

28. The method according to claim 26 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

29. The method according to claim 18 wherein said pathological condition is epilepsy, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the epilepsy.

30. The method according to claim 29 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

31. A The method according to claim 18 wherein said pathological condition is a drug or alcohol addiction, said method comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the addiction.

32. The method according to claim 31 wherein said oxidizing agent is selected from the group consisting of ebselen, selenocystine, selenocystamine, selenoglutathione disulfide, iron(III) chelates, dehydroascorbate, glutathione disulfide, coenzyme A disulfide, cystamine, FAD, NAD^+, copper (II) chelates, and combinations thereof.

33. The method according to claim 18 wherein said pathological condition is a severe mental illness, said method comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the severe mental illness.

34. The method according to claim 33 wherein said severe mental illness is selected from the group consisting of depression and schizophrenia.

35. The method according to claim 33 wherein said oxidizing agent is selected from the group consisting of ebselen, selenocystine, selenocystamine, selenoglutathione disulfide, iron(III) chelates, dehydroascorbate, glutathione disulfide, coenzyme A disulfide, cystamine, FAD, NAD^+, copper (II) chelates, and combinations thereof.

36. The method according to claim 18 wherein said pathological condition is an eating disorder.

37. The method according to claim 36 wherein said eating disorder is selected from the group consisting of anorexia nervosa, bulimia and obesity.

38. The method according to claim 18 wherein said pathological condition is anorexia nervosa, said method comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the anorexia nervosa.

39. The method according to claim 38 wherein said oxidizing agent is selected from the group consisting of ebselen, selenocystine, selenocystamine, selenoglutathione disulfide, iron(III) chelates, dehydroascorbate, glutathione disulfide, coenzyme A disulfide, cystamine, FAD, NAD^+, copper (II) chelates, and combinations thereof.

40. The method according to claim 18 wherein said pathological condition is obesity, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the obesity.

41. The method according to claim 40 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

42. The method according to claim 18 wherein said pathological condition is an inflammatory disease, said method comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the inflammatory disease wherein said inflammatory disease is selected from the group consisting of colitis and Crohn's disease.

43. The method according to claim 42 wherein said oxidizing agent is selected from the group consisting of ebselen, selenocystine, selenocystamine, selenoglutathione disulfide, iron(III) chelates, dehydroascorbate, glutathione disulfide, coenzyme A disulfide, cystamine, FAD, NAD^+, copper (II) chelates, and combinations thereof.

44. The method according to claim 18 wherein said pathological condition is a disorder of the endocrine system, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the disorder of the endocrine system.

45. The method according to claim 44 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

46. The method according to claim 44 wherein said disorder of the endocrine system is selected from prostate cancer, prostate hypertrophy, a disorder of the thyroid, a disorder of the ovaries, and a disorder of the adrenal gland.

47. The method according to claim 18 wherein said pathological condition is pathological apoptosis, said method comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the pathological apoptosis.

48. The method according to claim 47 wherein said oxidizing agent is selected from the group consisting of ebselen, selenocystine, selenocystamine, selenoglutathione disulfide, iron(III) chelates, dehydroascorbate, glutathione disulfide, coenzyme A disulfide, cystamine, FAD, NAD^+, copper (II) chelates, and combinations thereof.

49. The method according to claim 18 wherein said pathological condition is cancer, said method comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for inducing apoptosis of the cells of the cancer.

50. The method according to claim 49 wherein said oxidizing agent is selected from the group consisting of ebselen, selenocystine, selenocystamine, selenoglutathione disulfide, iron(III) chelates, dehydroascorbate, glutathione disulfide, coenzyme A disulfide, cystamine, FAD, NAD^+, copper (II) chelates, and combinations thereof.

51. The method according to claim 18 wherein said pathological condition is cancer, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for inhibiting proliferation of the cells of the cancer.

52. The method according to claim 51 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

53. The method according to claim 18 wherein said pathological condition is an autoimmune disease, said method comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the autoimmune disease.

54. The method according to claim 53 wherein said oxidizing agent is selected from the group consisting of ebselen, selenocystine, selenocystamine, selenoglutathione disulfide, iron(III) chelates, dehydroascorbate, glutathione disulfide, coenzyme A disulfide, cystamine, FAD, NAD^+, copper (II) chelates, and combinations thereof.

55. The method according to claim 18 wherein said pathological condition is a viral disease.

56. The method according to claim 18 wherein said viral disease is selected from the group consisting of infection by the common cold, HIV, hepatitis C, measles, papilloma, and Semliki Forest virus.

57. The method according to claim 18 wherein said pathological condition is an infection with HIV, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the infection with HIV.

58. The method according to claim 57 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II)

chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

59. The method according to claim 18 wherein said pathological condition is a plant disease.

60. The method according to claim 59 wherein said plant disease is ilarvirus or mosaic virus, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein amount effective for treatment of the infection with the ilarvirus or the mosaic virus.

61. The method according to claim 60 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

62. The method according to claim 18 wherein said pathological condition is infection with papilloma virus, said method comprising administering to the subject an oxidizing agent that promotes release of zinc from metallothionein in an amount effective for treatment of the papilloma virus infection.

63. The method according to claim 62 wherein said oxidizing agent is selected from the group consisting of ebselen, selenocystine, selenocystamine, selenoglutathione disulfide, iron(III) chelates, dehydroascorbate, glutathione disulfide, coenzyme A disulfide, cystamine, FAD, NAD^+, copper (II) chelates, and combinations thereof.

64. The method according to claim 18 wherein said pathological condition is infection with hepatitis C virus, said method comprising administering to the subject a reducing agent that inhibits release of zinc from metallothionein in an amount effective for treatment of the hepatitis C virus infection.

65. The method according to claim 64 wherein said reducing agent is selected from the group consisting of selenocysteine, selenoglutathione, selenocysteamine, iron(II) chelates, ascorbate, glutathione, coenzyme A, cysteamine, $FADH_2$, NADH, copper(I) chelates, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

66. The method according to claim 18 further comprising co-administering a cofactor selected from the group consisting of glutathione, ATP and GTP.

67. The method according to claim 18 wherein said compound is combined with one or more ingredients selected from the group consisting of a pharmaceutically acceptable carrier, a pharmaceutically acceptable excipient, a wetting agent, a buffering agent, an emulsifying agent, and a binding agent.

68. The method according to claim 18 wherein said compound is formulated as a pharmaceutically acceptable salt.

69. The method according to claim 18 wherein said compound is purified.

70. The method according to claim 18 further comprising coadministering with said compound an amount of purified metallothionein or a derivative thereof.

71. The method according to claim 70 wherein a metallothionein derivative is coadministered, said derivative being bound to an antibody to a cellular antigen.

72. The method according to claim 18 wherein said compound is adapted for intravenous administration to human beings.

73. A pharmaceutical composition comprising purified glutathione in combination with a purified compound selected from the group consisting of ebselen, cytochrome c, ascorbate, dehydroascorbate, glutathione disulfide, coenzyme A, cystamine, cysteamine, FAD, $FADH_2$, azurin, vitamin E, N-acetyl-L-cysteine, α-lipoate, and combinations thereof.

74. A kit comprising in one or more containers purified glutathione and a purified compound selected from the group consisting of cytochrome c, ascorbate, glutathione, glutathione disulfide, coenzyme A, cystamine, cysteamine, FAD, $FADH_2$, azurin, N-acetyl-L-cysteine, α-lipoate, and combinations thereof, in an amount effective to modulate the release of zinc from metallothionein.

75. A kit comprising in one or more containers a purified compound selected from the group consisting of cytochrome c, glutathione, glutathione disulfide, coenzyme A, cystamine, cysteamine, FAD, $FADH_2$, azurin, N-acetyl-L-cysteine, α-lipoate, and combinations thereof, in an amount effective to modulate the release of zinc from metallothionein.

76. The method according to claim 18 wherein said compound is formulated as a suppository.

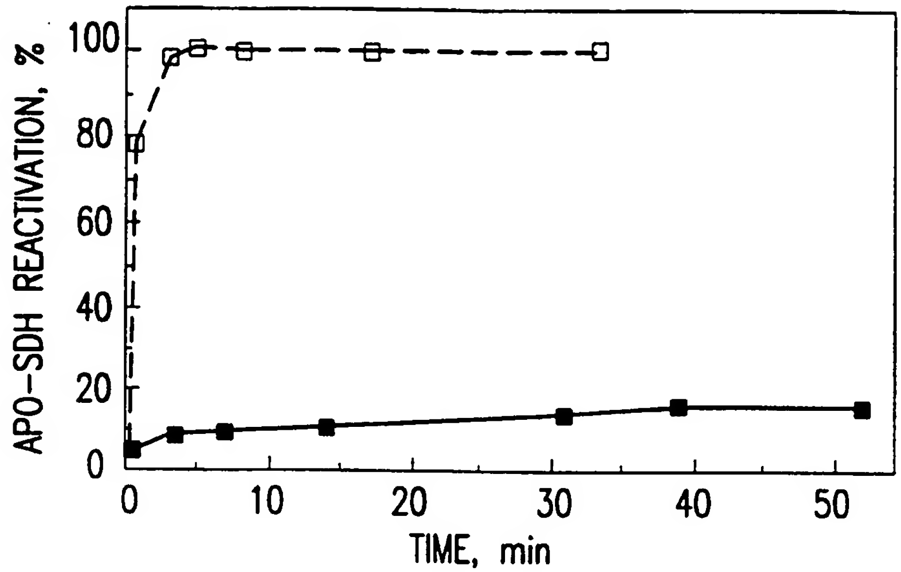

APO-SDH REACTIVATION, %
100
80
60
40
20
0
0
10
20
30
40
50
TIME, min

FIG.1

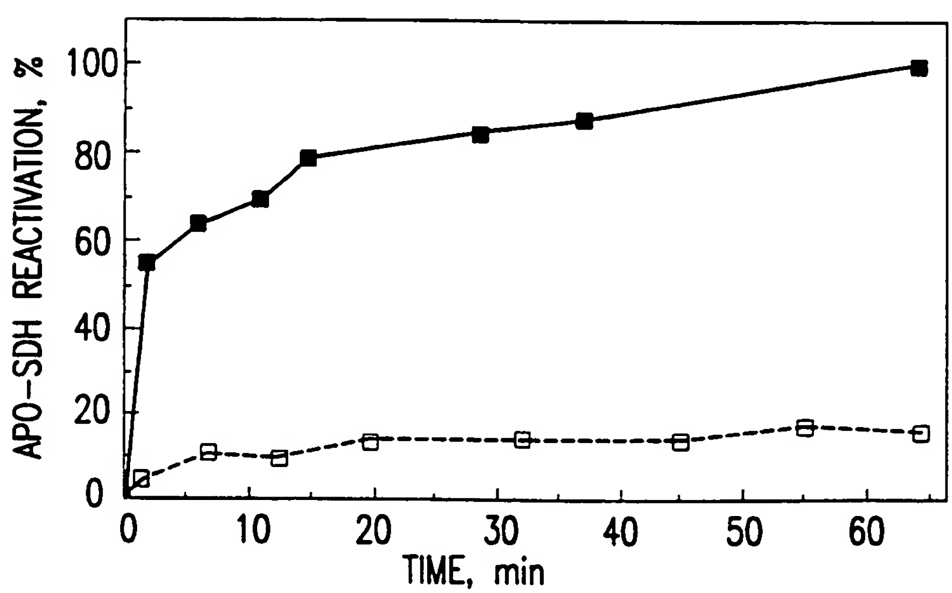

APO-SDH REACTIVATION, %
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME, min

FIG.2

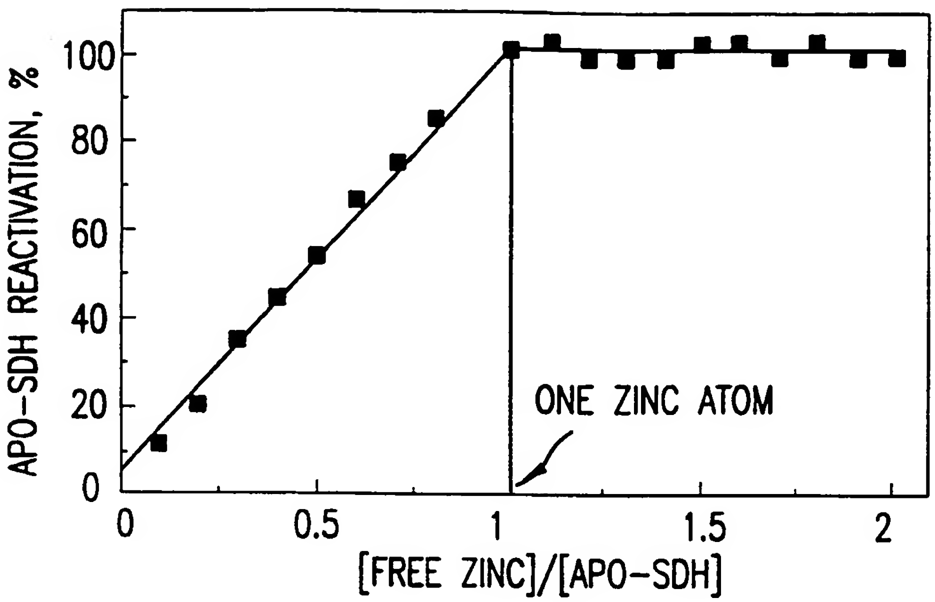

APO-SDH REACTIVATION, %
100
80
60
40
20
0
ONE ZINC ATOM
0
0.5
1
1.5
2
[FREE ZINC]/[APO-SDH]

FIG.3A

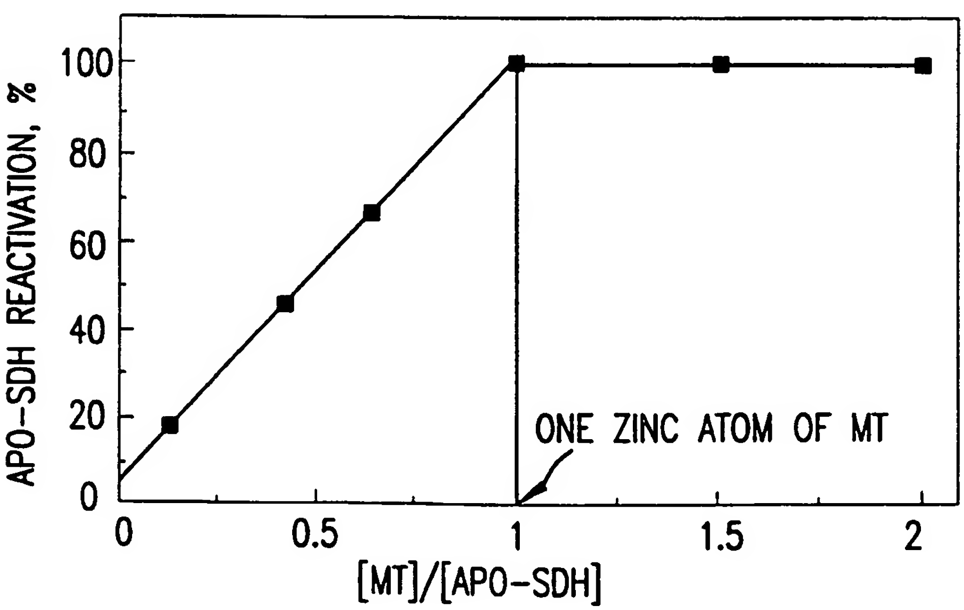

APO-SDH REACTIVATION, %
100
80
60
40
20
0
ONE ZINC ATOM OF MT
0
0.5
1
1.5
2
[MT]/[APO-SDH]

FIG.3B

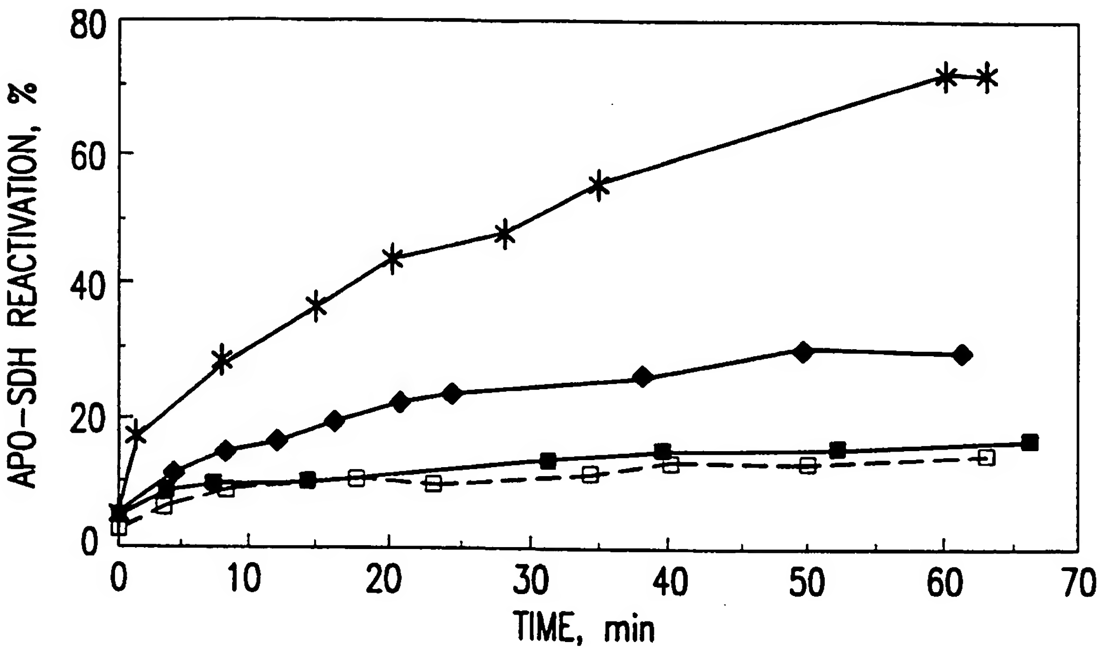


FIG.4

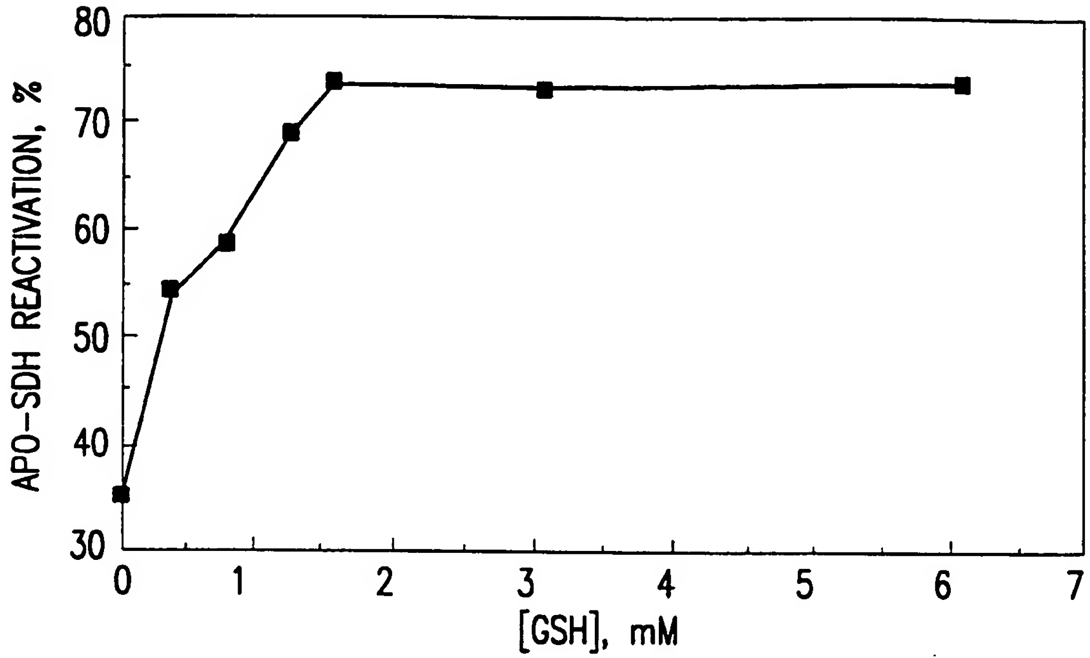

APO-SDH REACTIVATION, %
80
70
60
50
40
30
0
1
2
3
4
5
6
7
[GSH], mM

FIG.5A

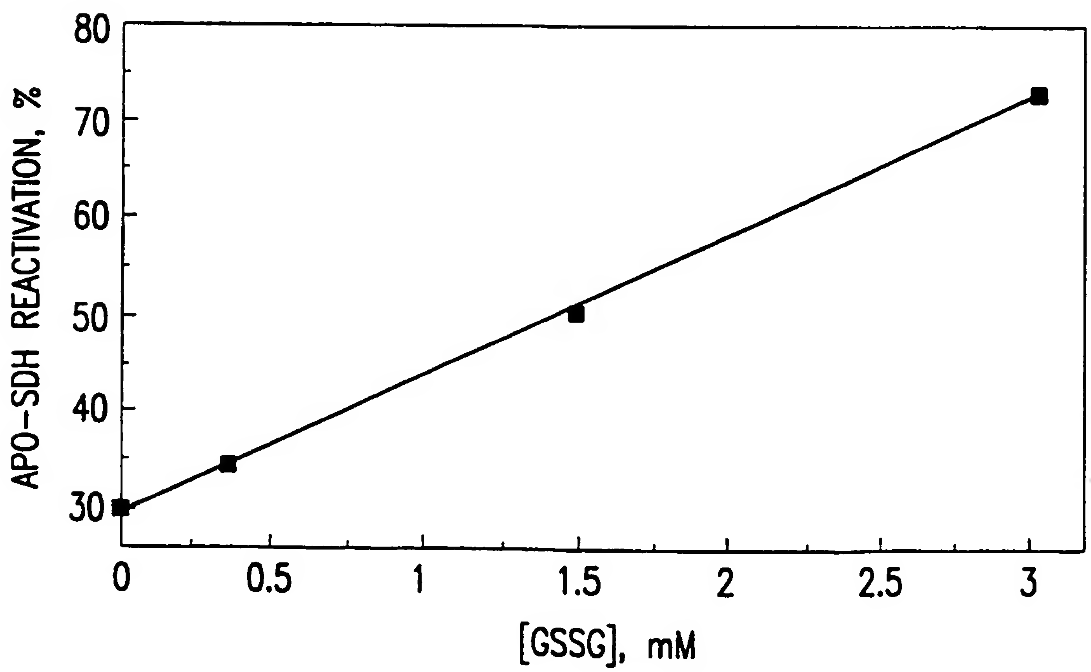

APO-SDH REACTIVATION, %
80
70
60
50
40
30
0
0.5
1
1.5
2
2.5
3
[GSSG], mM

FIG.5B

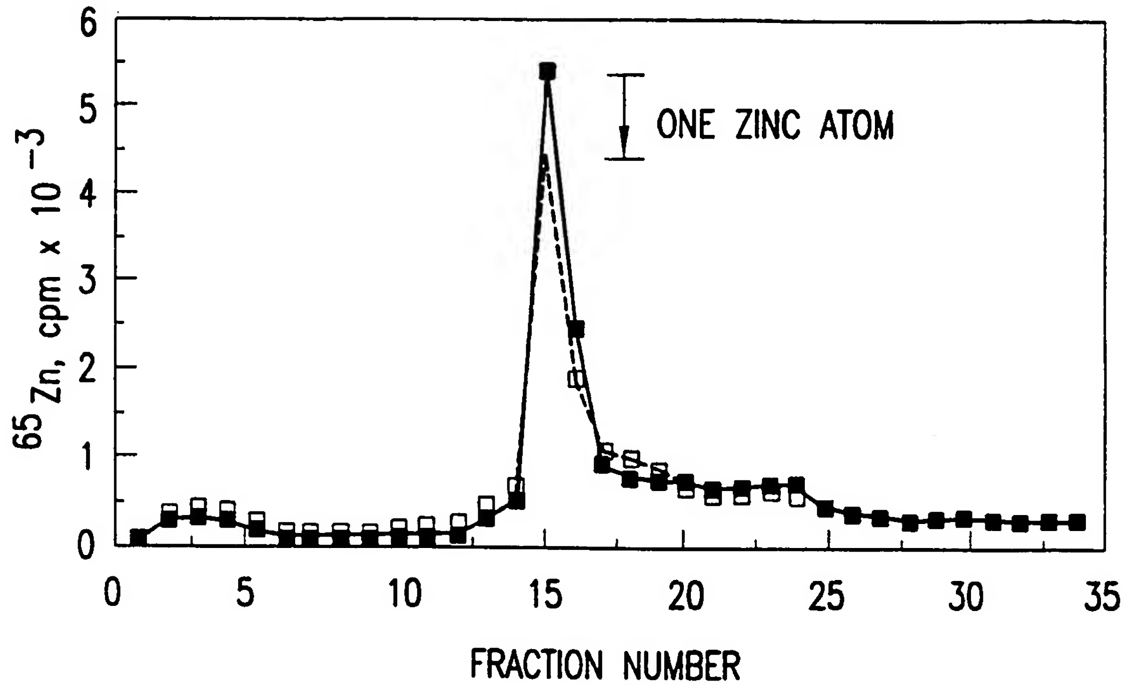

6
5
4
3
2
1
0
^{65}Zn, cpm x 10^{-3}
ONE ZINC ATOM
0
5
10
15
20
25
30
35
FRACTION NUMBER

FIG.6A

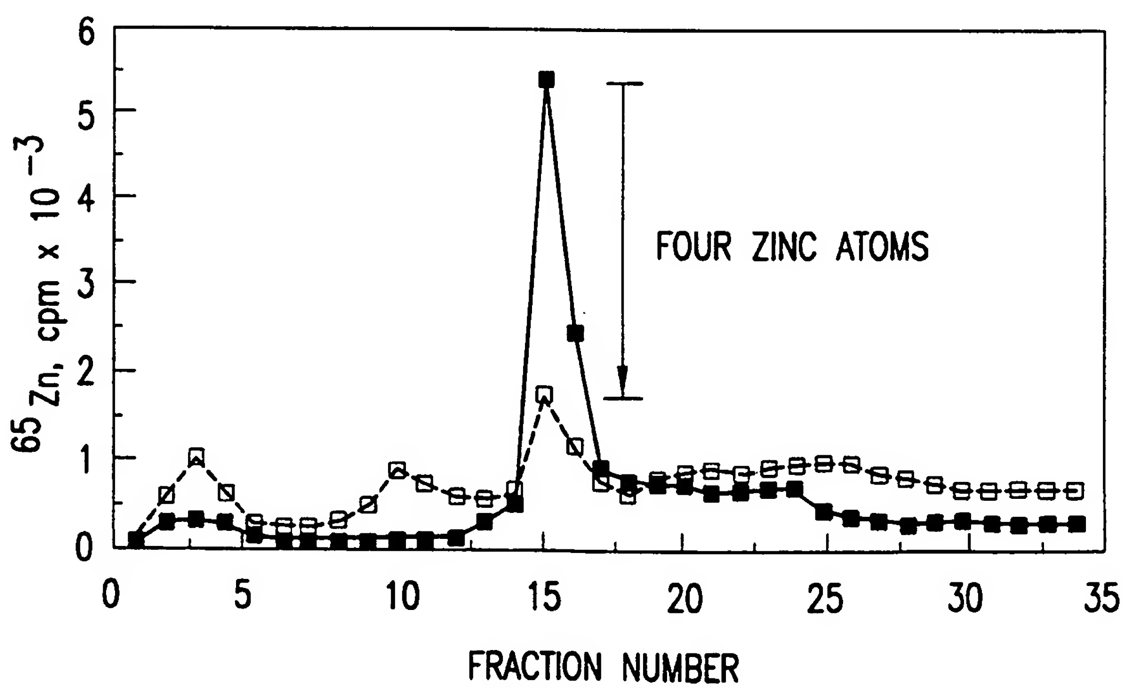

6
5
4
3
2
1
0
^{65}Zn, cpm x 10^{-3}
FOUR ZINC ATOMS
0
5
10
15
20
25
30
35
FRACTION NUMBER

FIG.6B

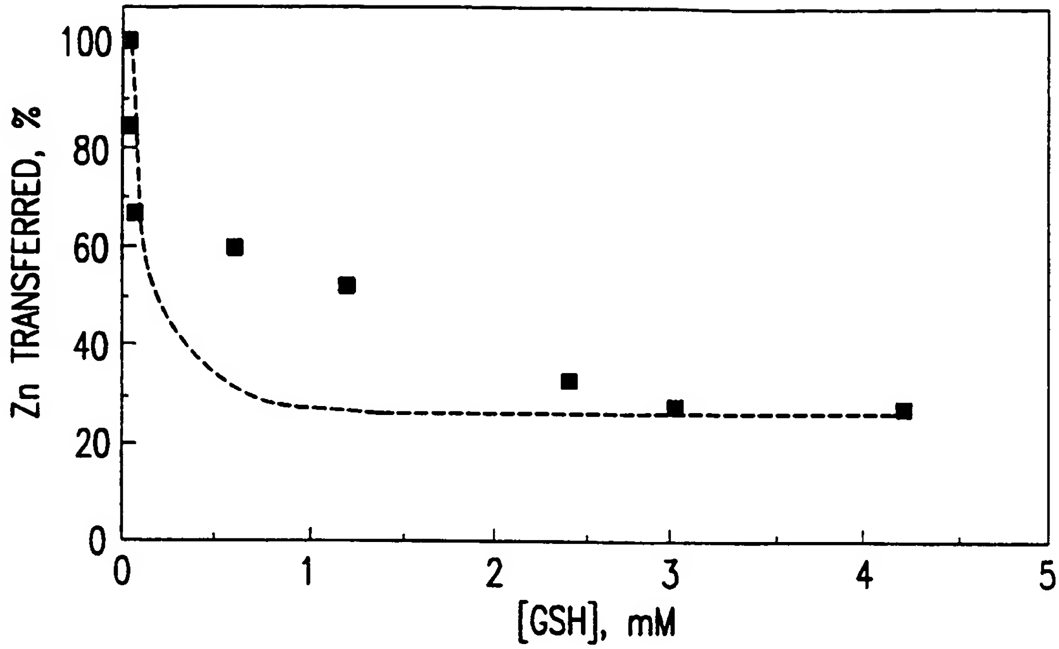

Zn TRANSFERRED, %
0
20
40
60
80
100
0
1
2
3
4
5
[GSH], mM

FIG.7

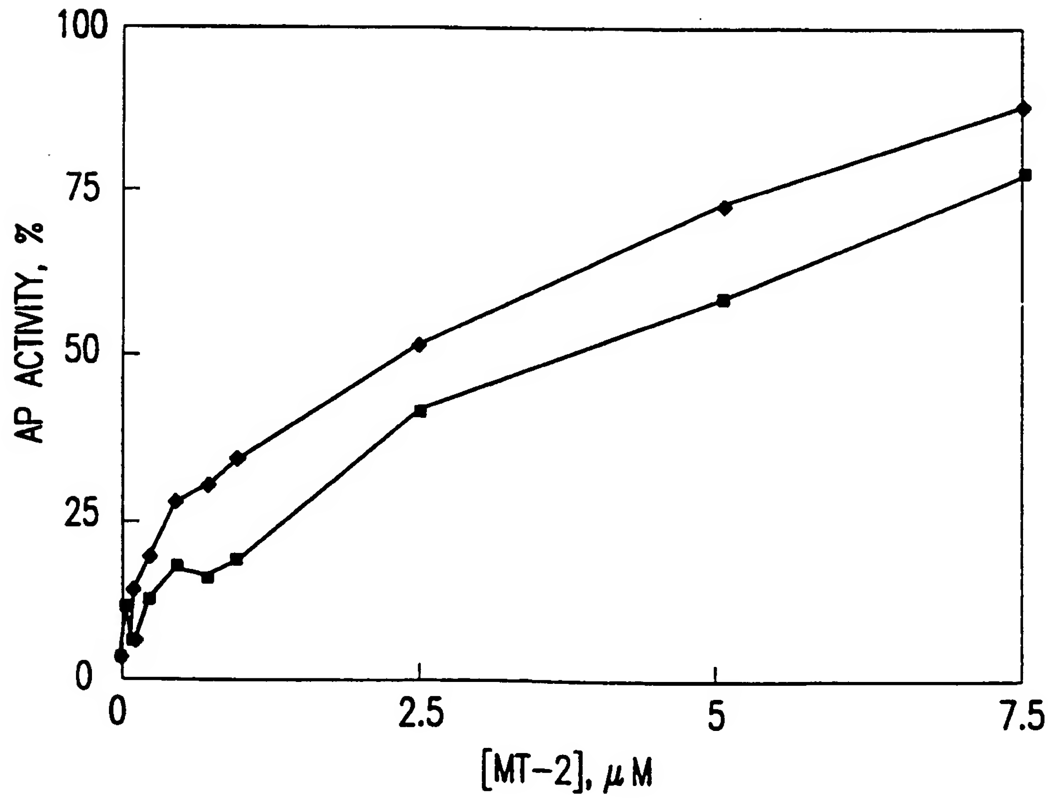

AP ACTIVITY, %
0
25
50
75
100
0
2.5
5
7.5
[MT-2], μM

FIG.8

7/24

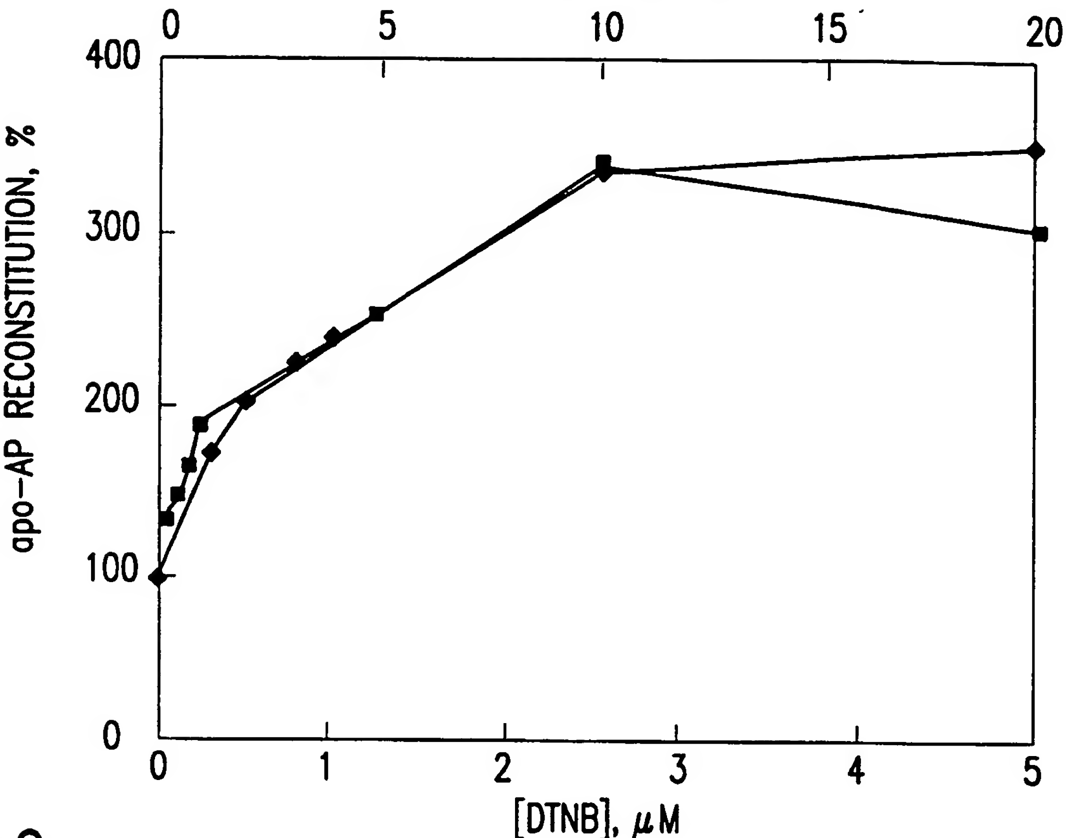


FIG.9

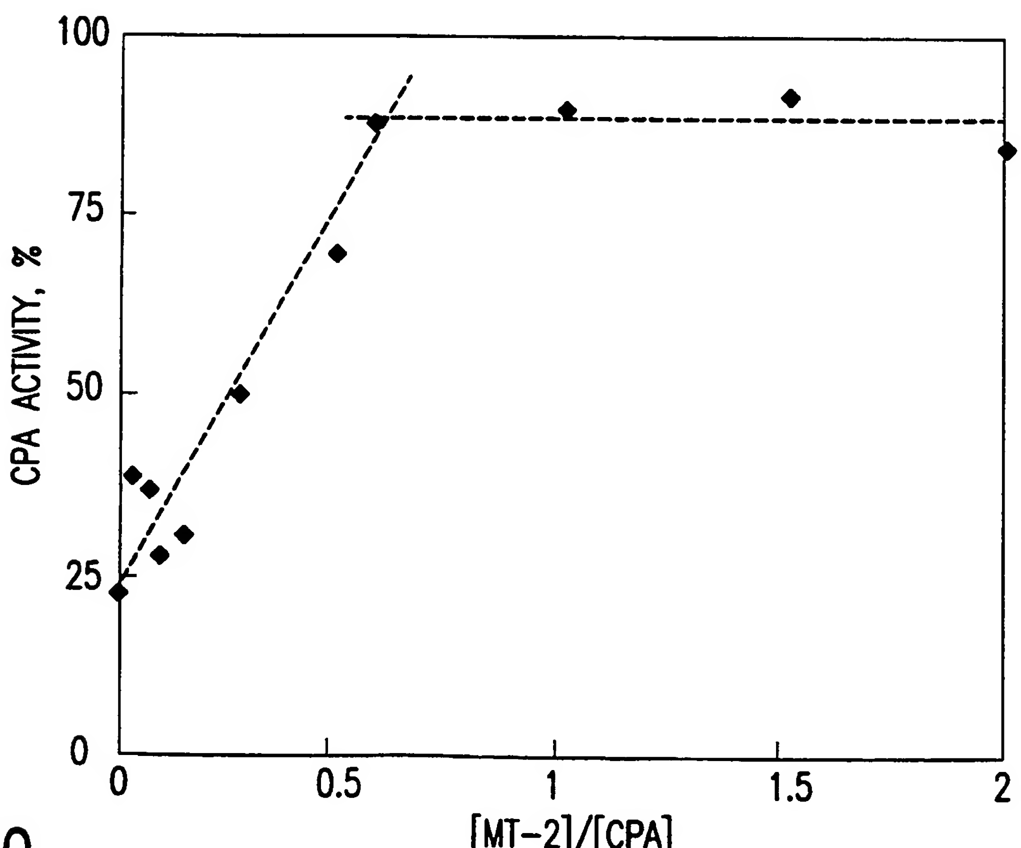


FIG.10

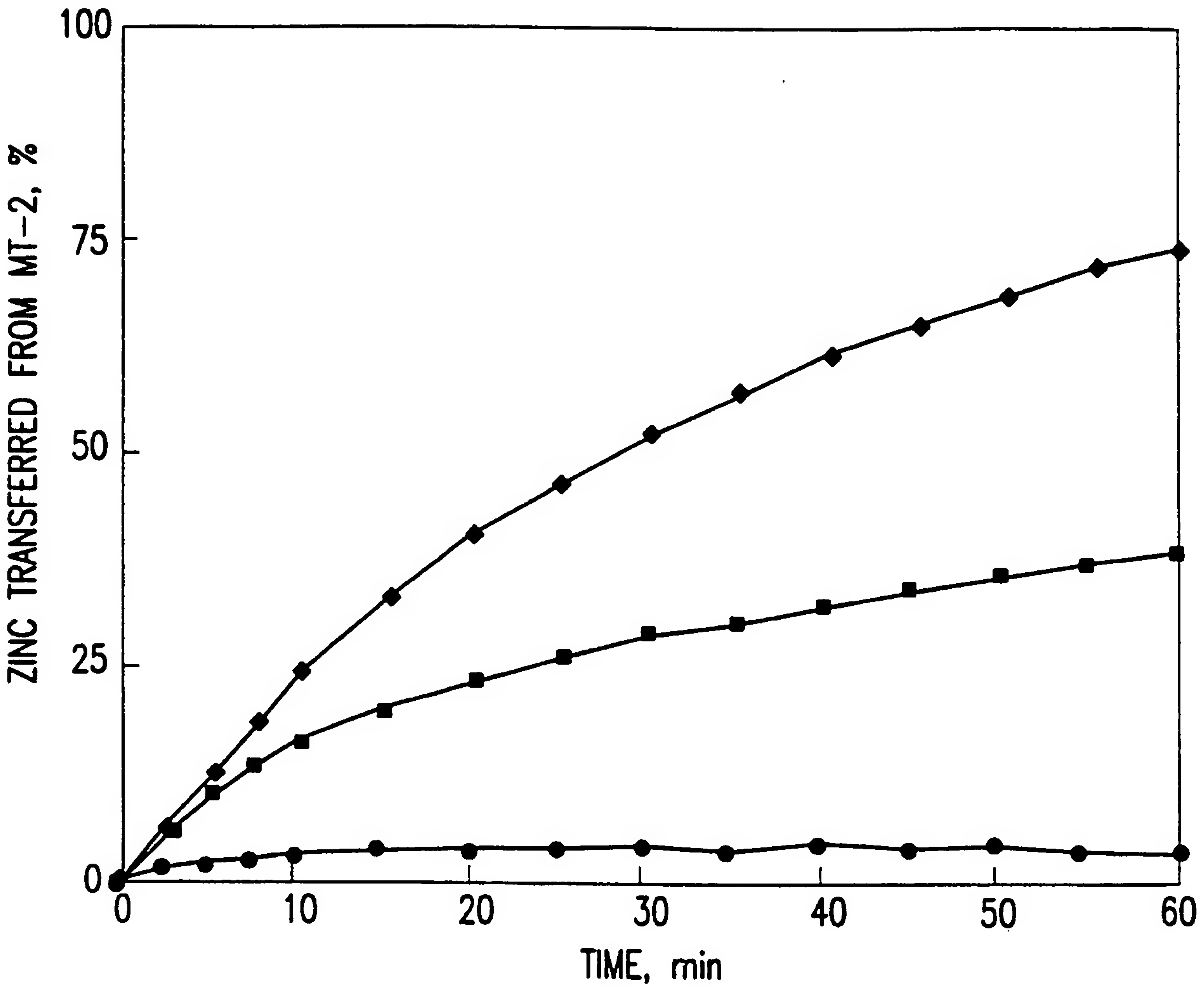

100
75
50
25
0
ZINC TRANSFERRED FROM MT-2, %
0
10
20
30
40
50
60
TIME, min

FIG.11

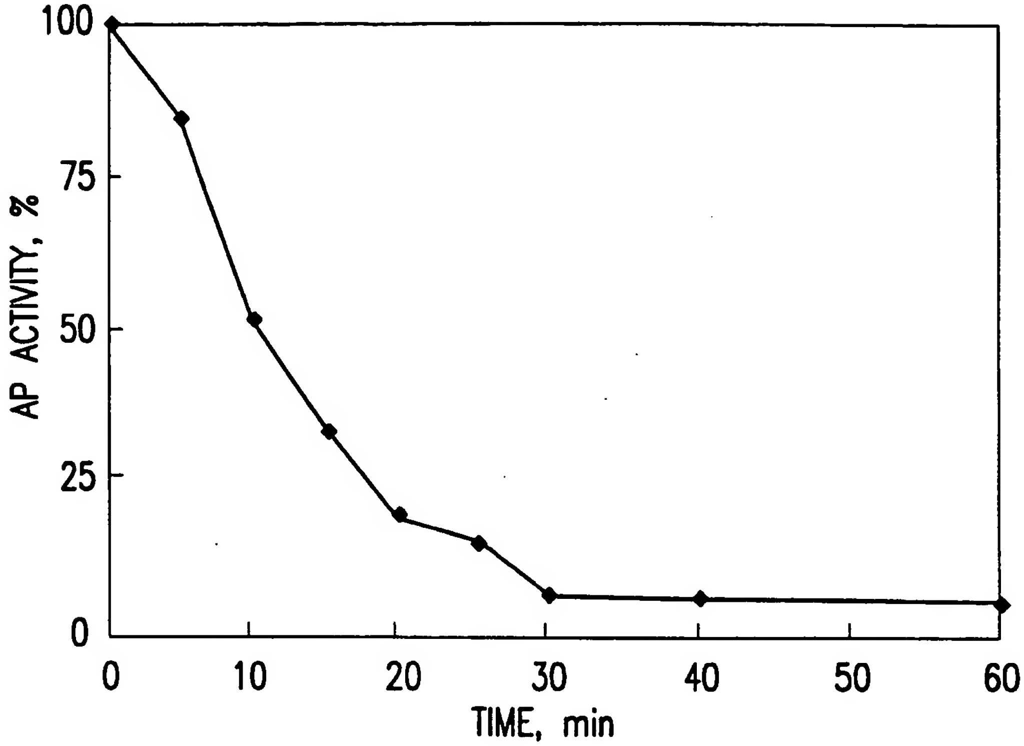

100
75
50
25
0
AP ACTIVITY, %
0
10
20
30
40
50
60
TIME, min

FIG.12

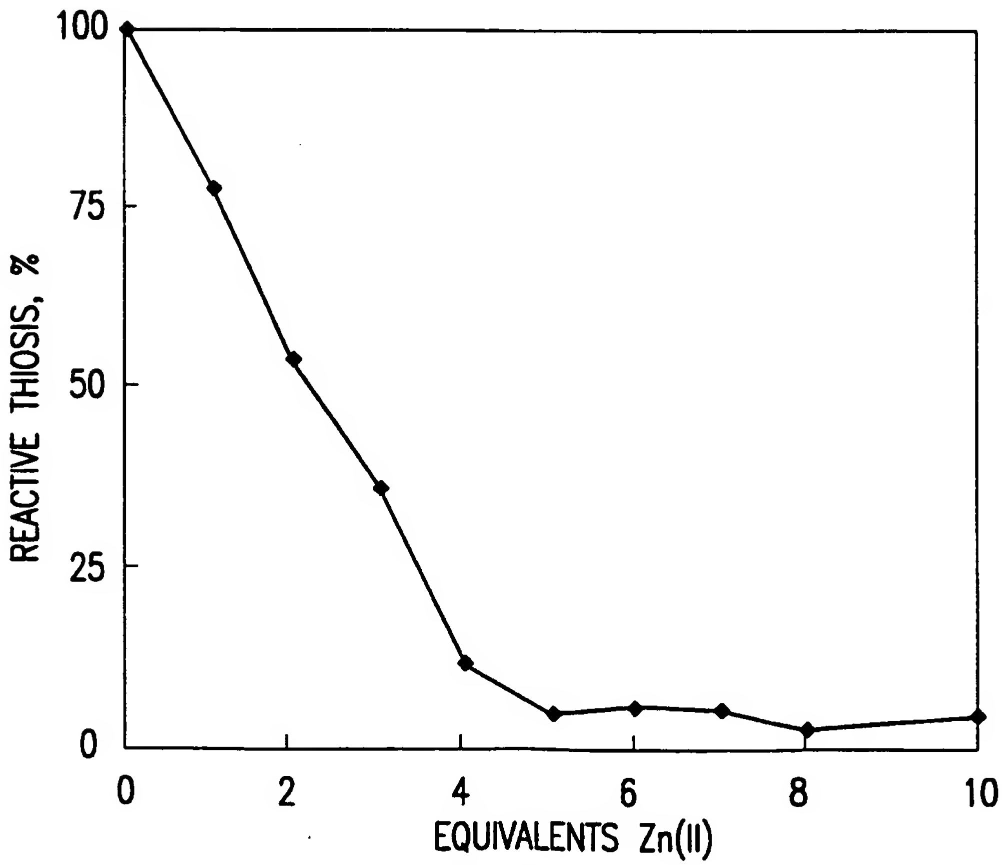

100
75
50
25
0
REACTIVE THIOSIS, %
0
2
4
6
8
10
EQUIVALENTS Zn(II)

FIG.13

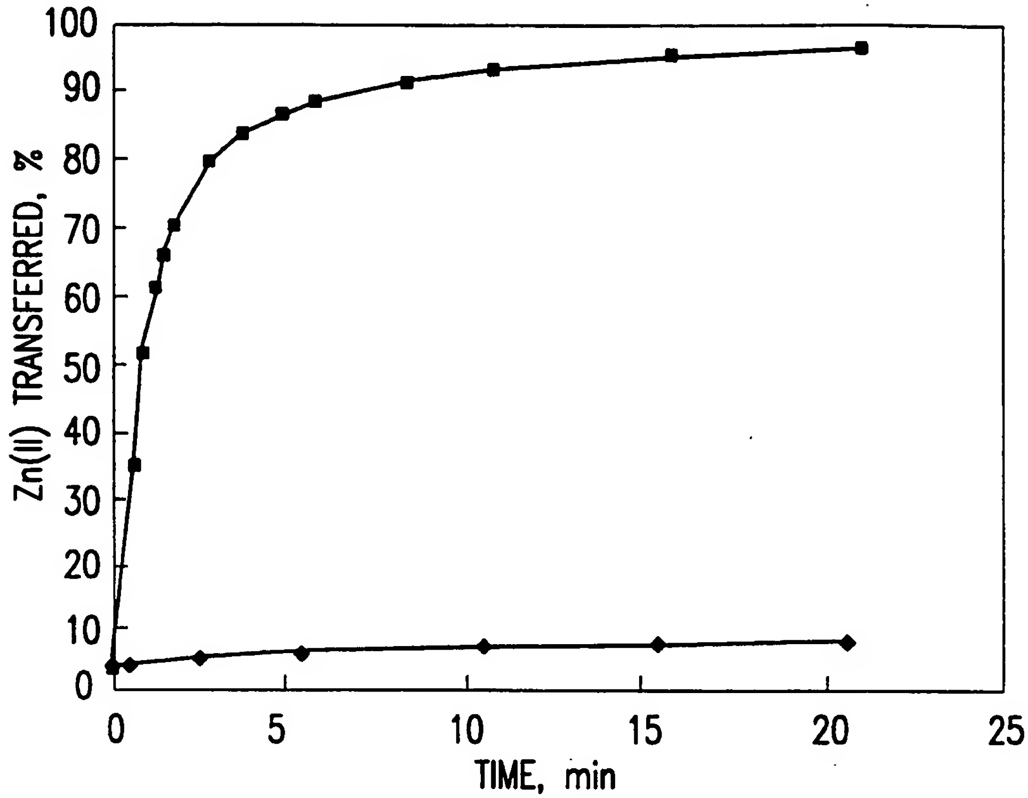


FIG.14A

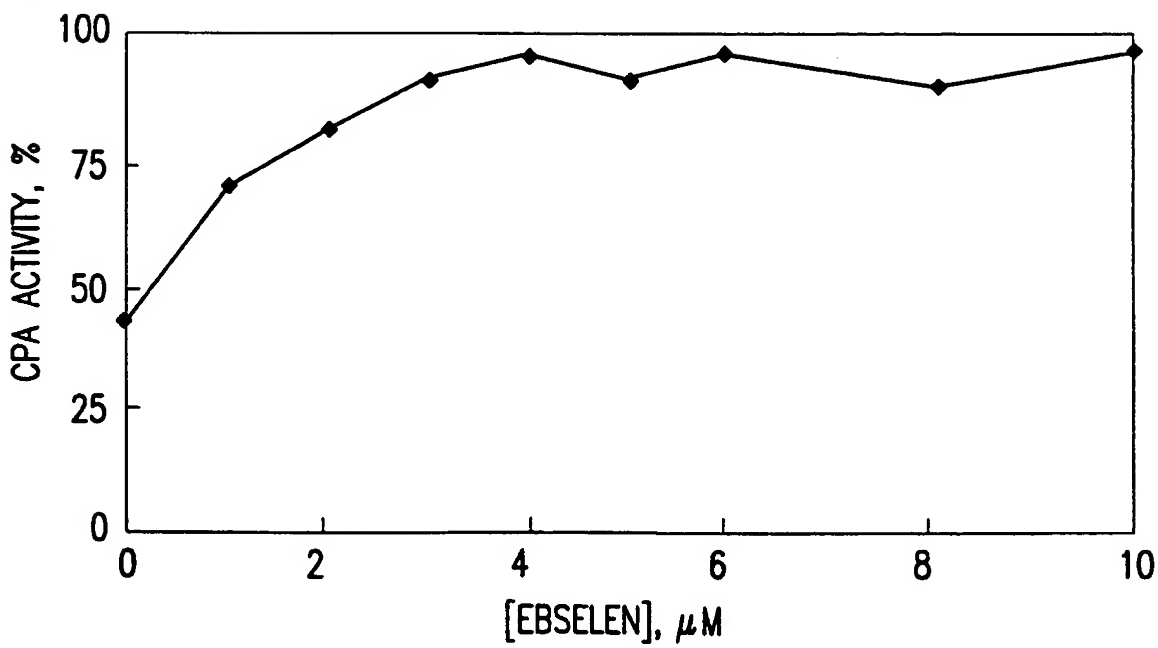


FIG.14B

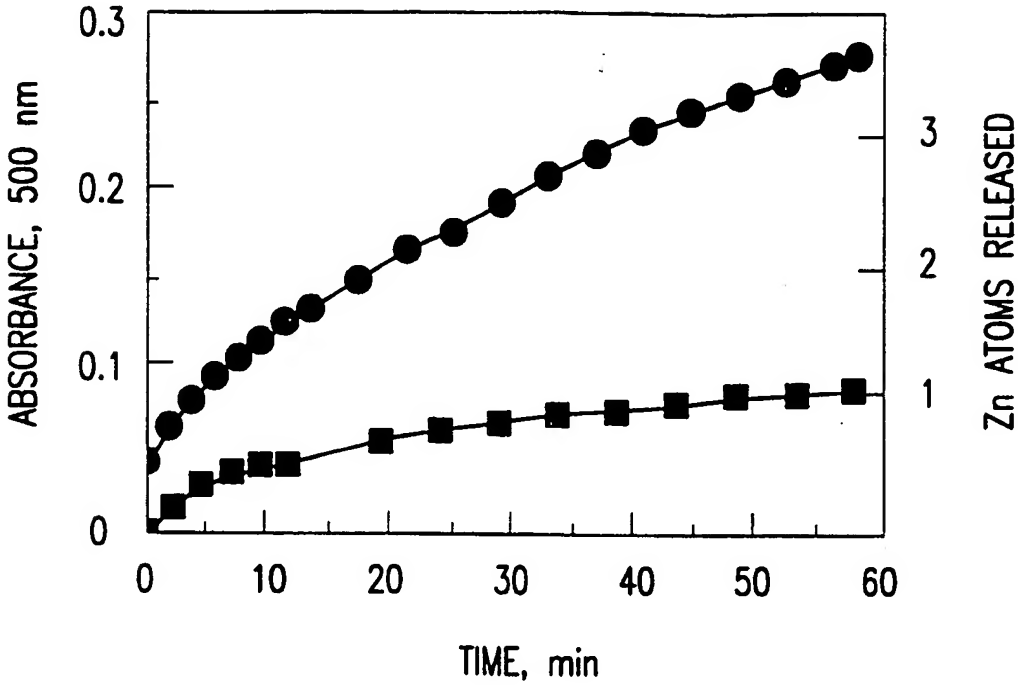

0.3
0.2
0.1
0
ABSORBANCE, 500 nm
3
2
1
Zn ATOMS RELEASED
0
10
20
30
40
50
60
TIME, min

FIG.15

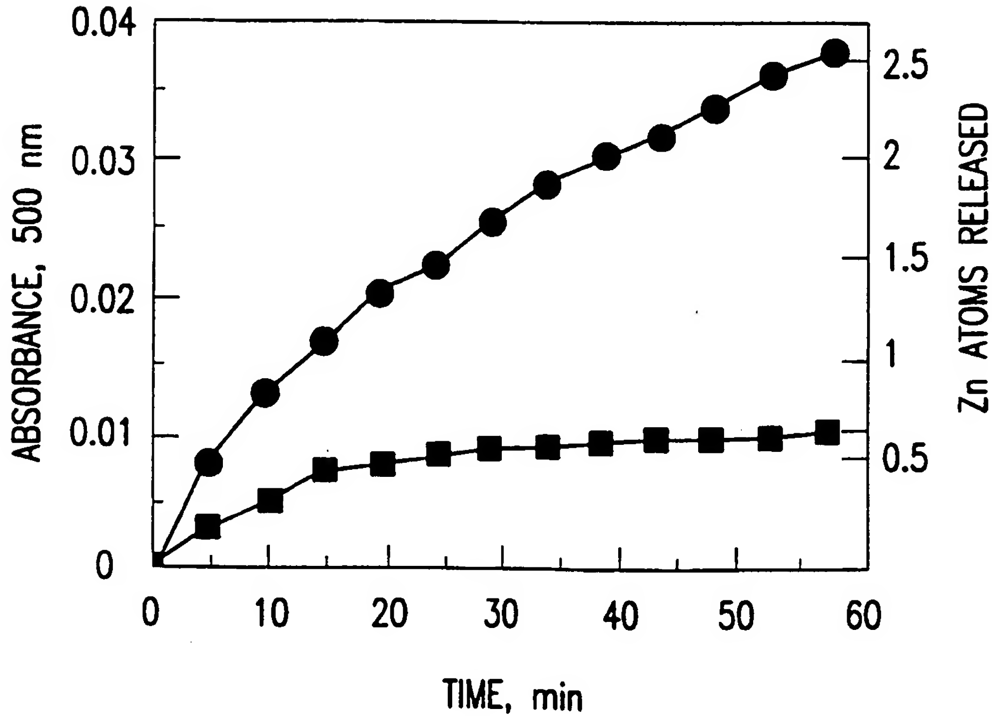
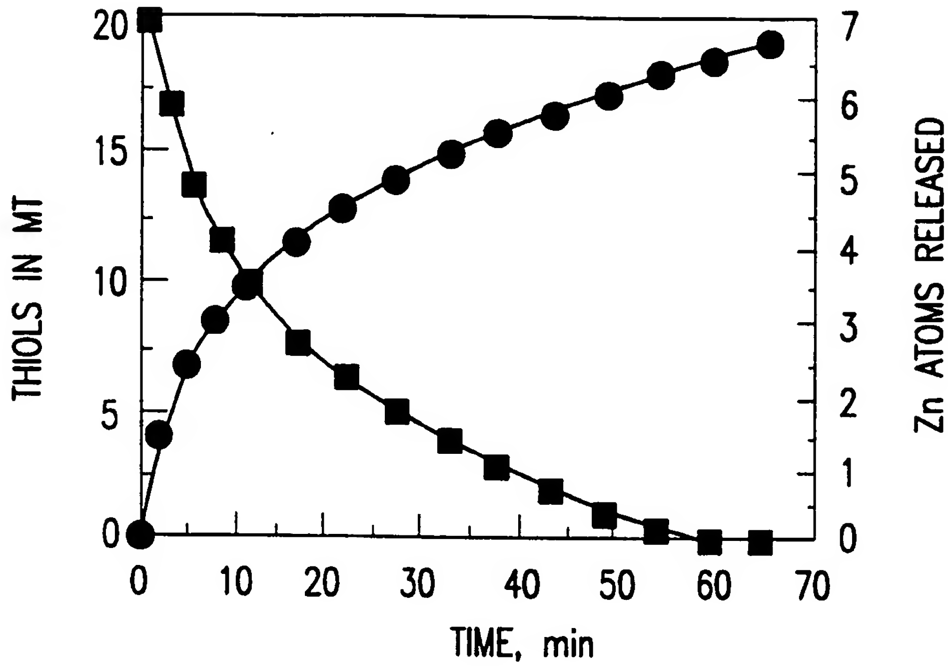
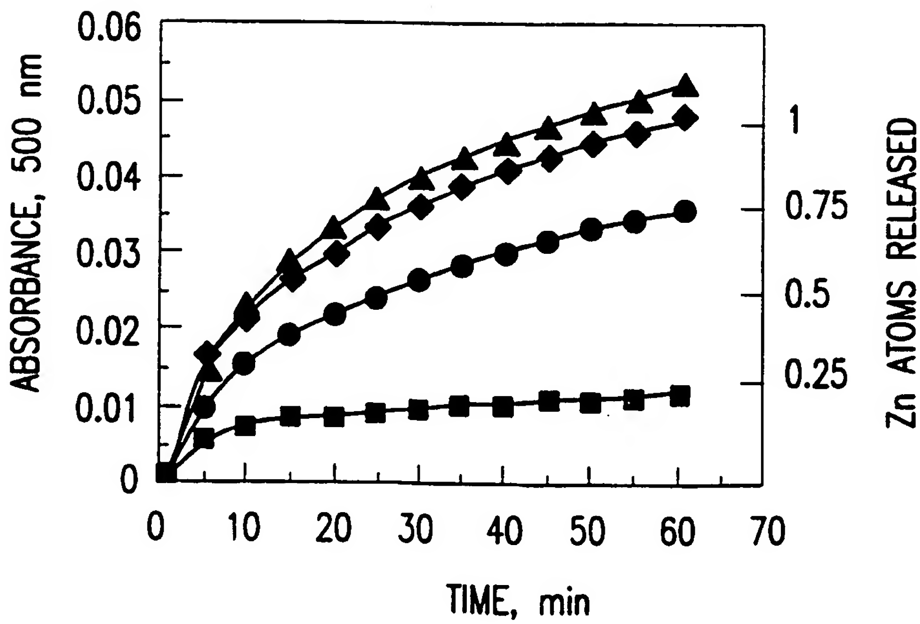

0.04
0.03
0.02
0.01
0
ABSORBANCE, 500 nm
2.5
2
1.5
1
0.5
Zn ATOMS RELEASED
0
10
20
30
40
50
60
TIME, min

FIG.16



20
15
10
5
0
0
10
20
30
40
50
60
70
7
6
5
4
3
2
1
0
THIOLS IN MT
Zn ATOMS RELEASED
TIME, min

FIG.17



0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
1
0.75
0.5
0.25
ABSORBANCE, 500 nm
Zn ATOMS RELEASED
TIME, min

FIG.18

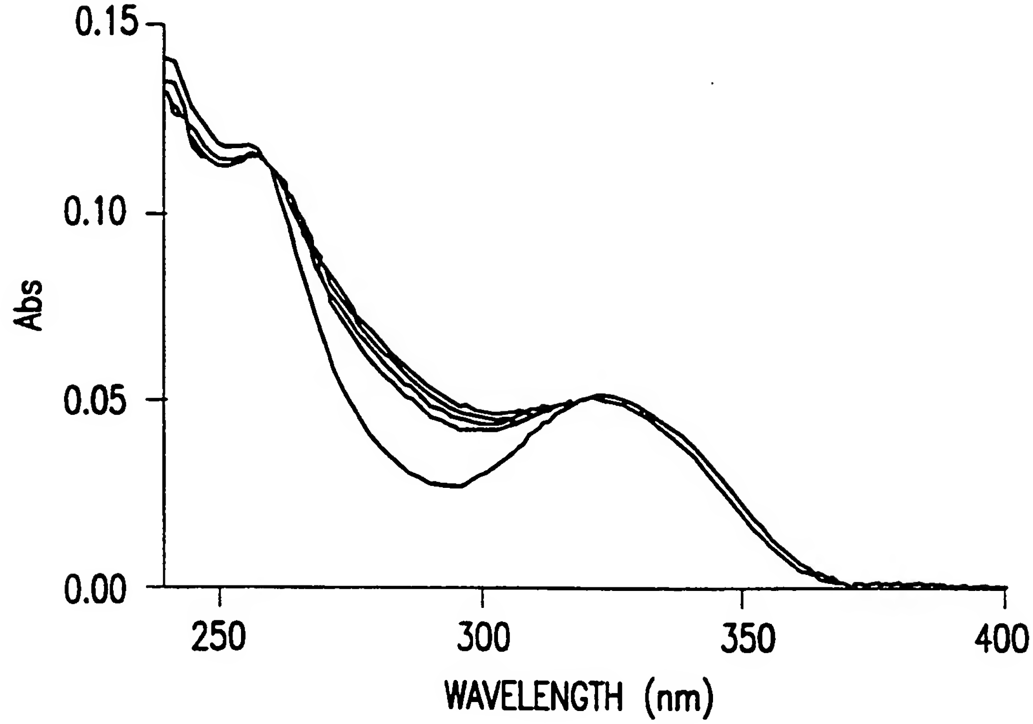


FIG.19A

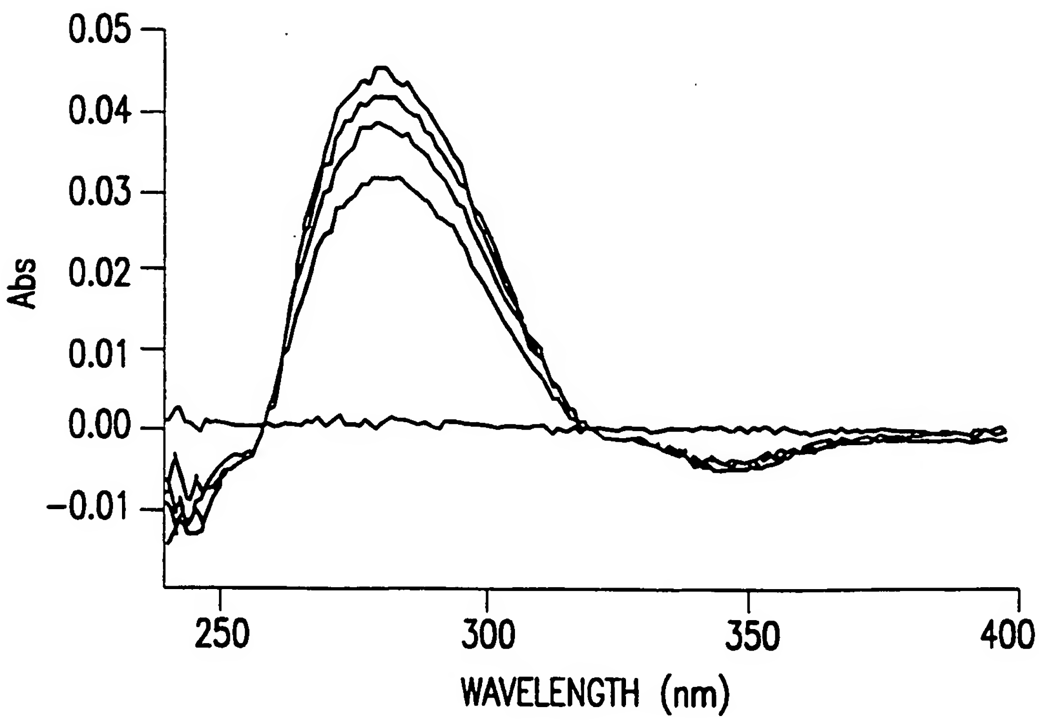


FIG.19B

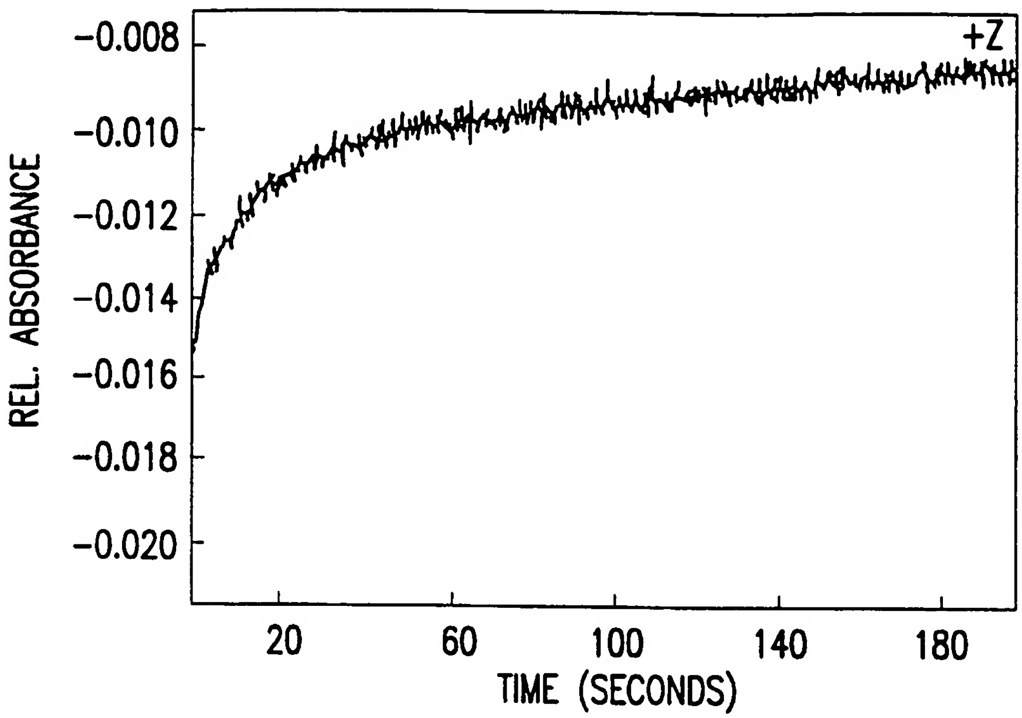


FIG.20A

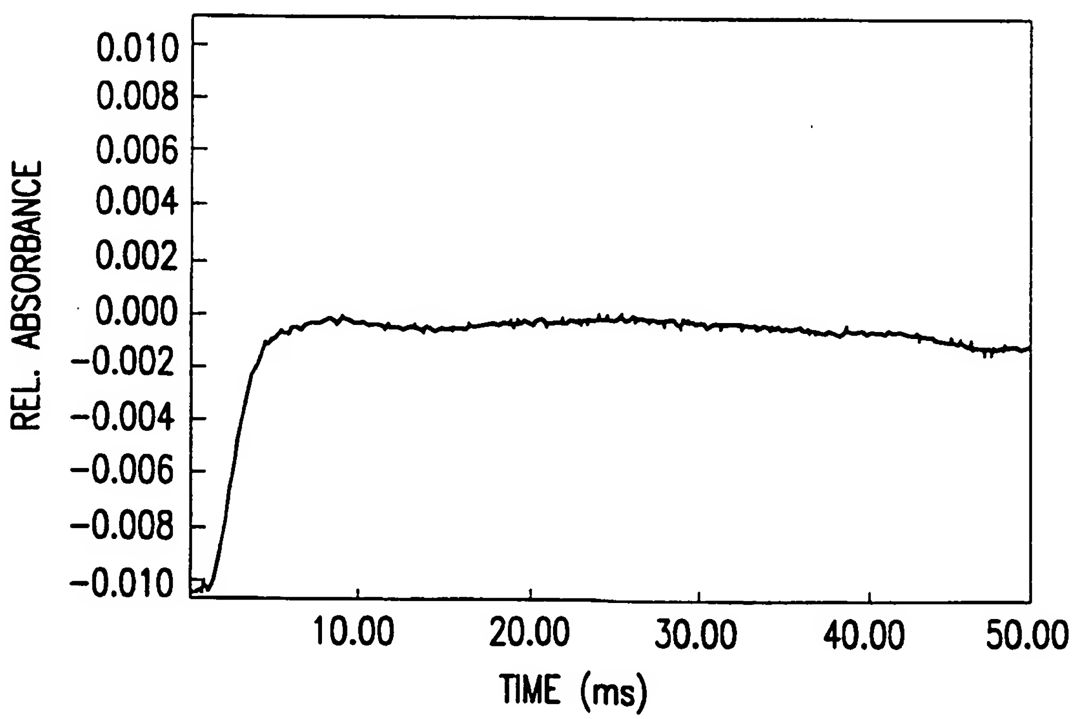


FIG.20B

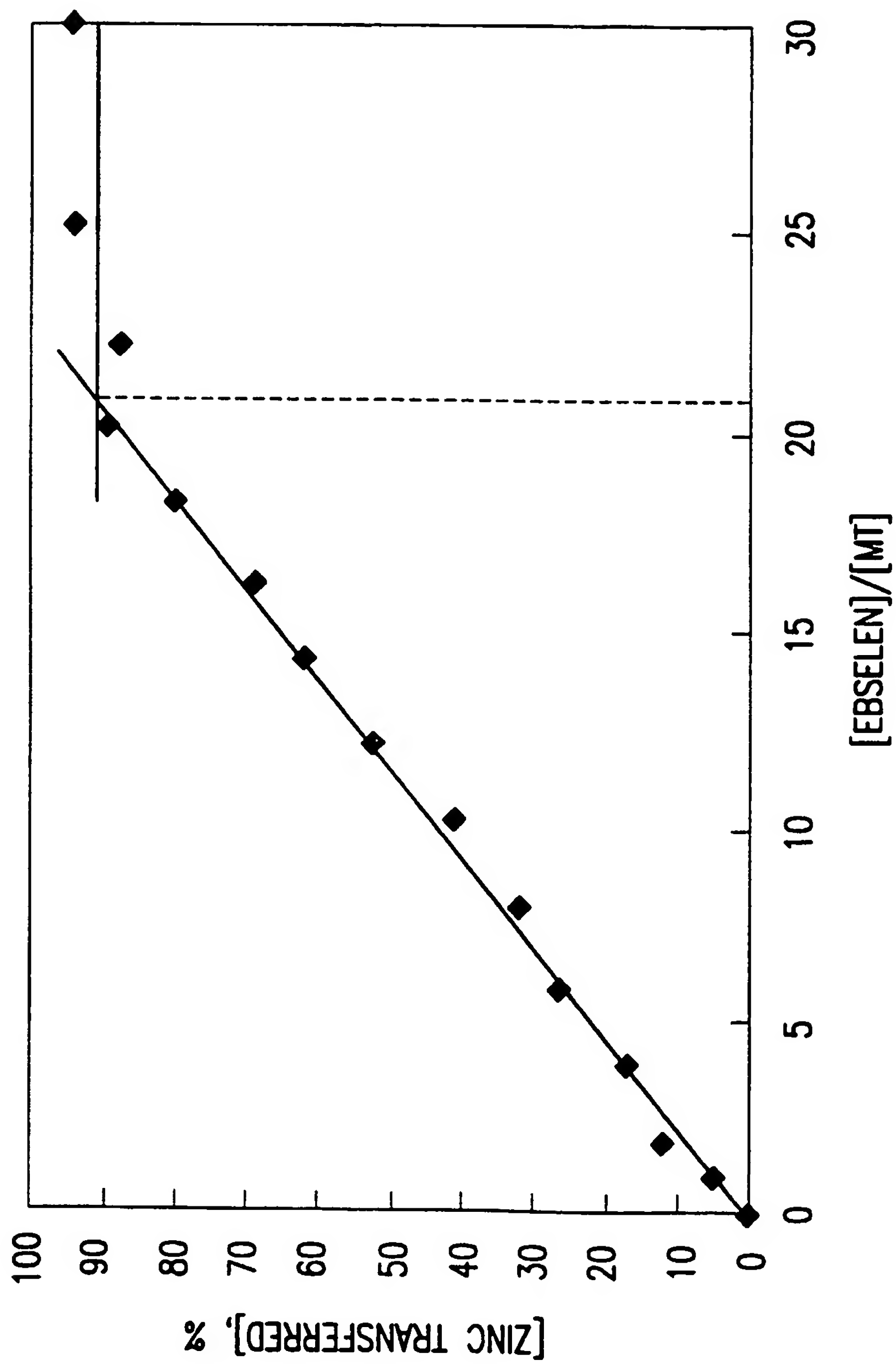

[ZINC TRANSFERRED], %
0
10
20
30
40
50
60
70
80
90
100
[EBSELEN]/[MT]
0
5
10
15
20
25
30

FIG.21

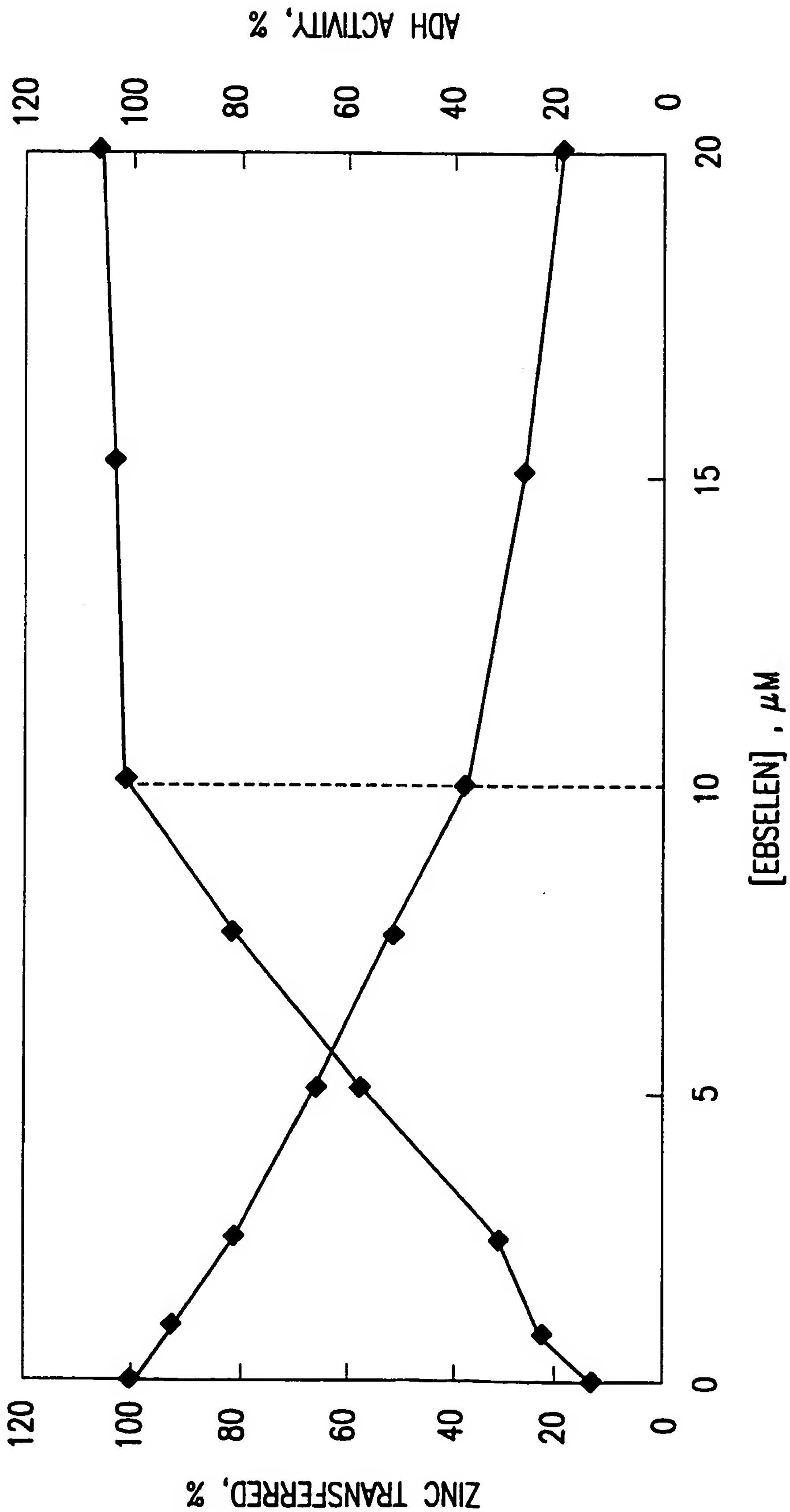

ADH ACTIVITY, %
120
100
80
60
40
20
0
ZINC TRANSFERRED, %
[EBSELEN] , μM
0
5
10
15
20

FIG.22

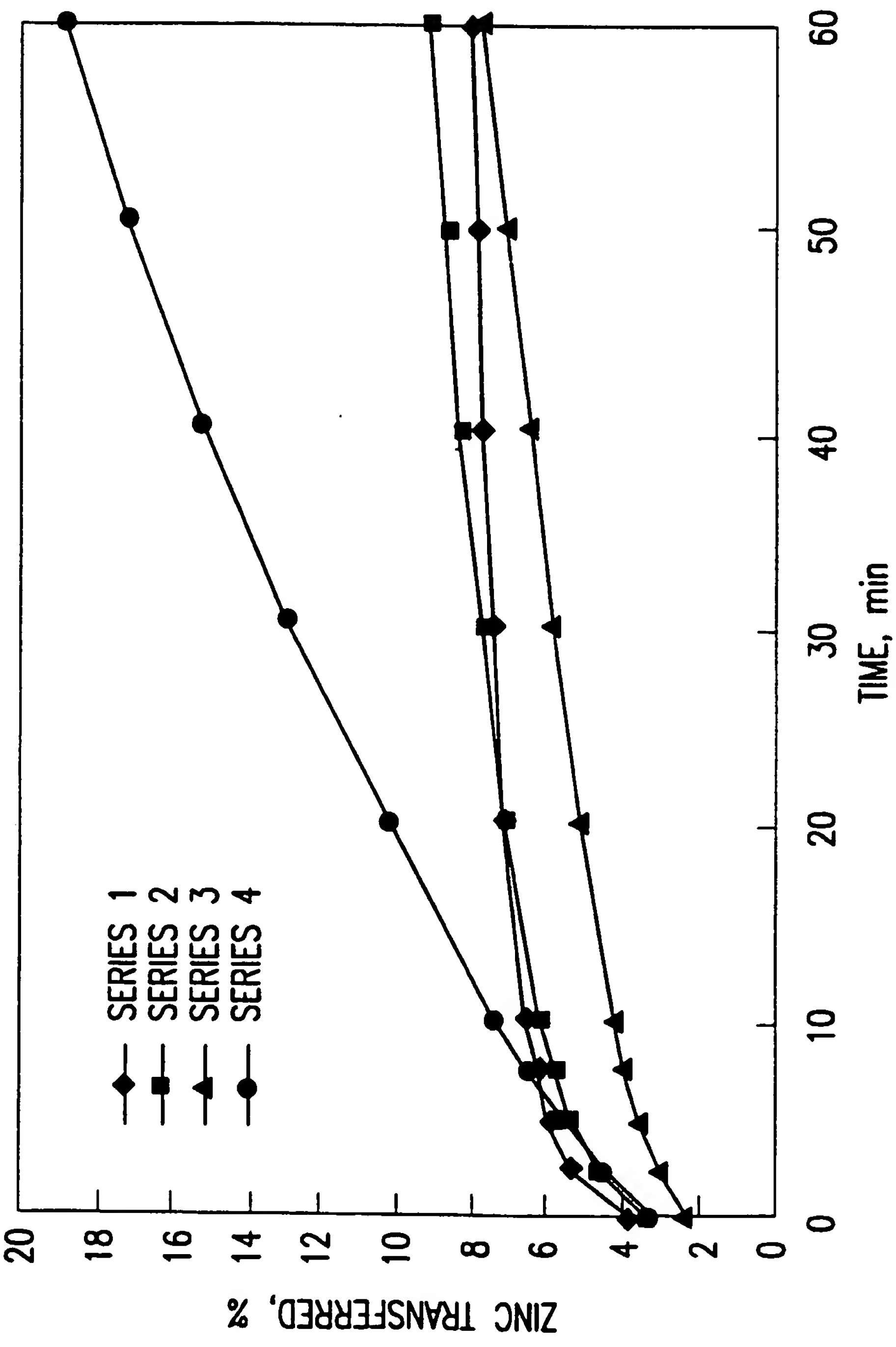

SERIES 1
SERIES 2
SERIES 3
SERIES 4
ZINC TRANSFERRED, %
TIME, min
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
40
50
60

FIG.23

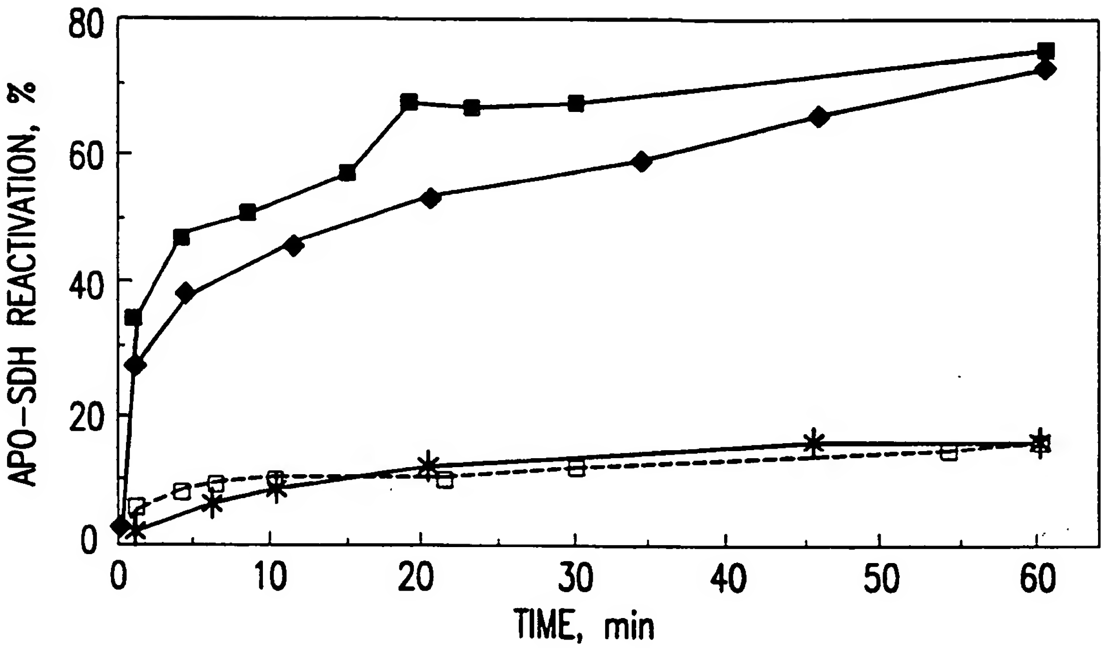


FIG.24

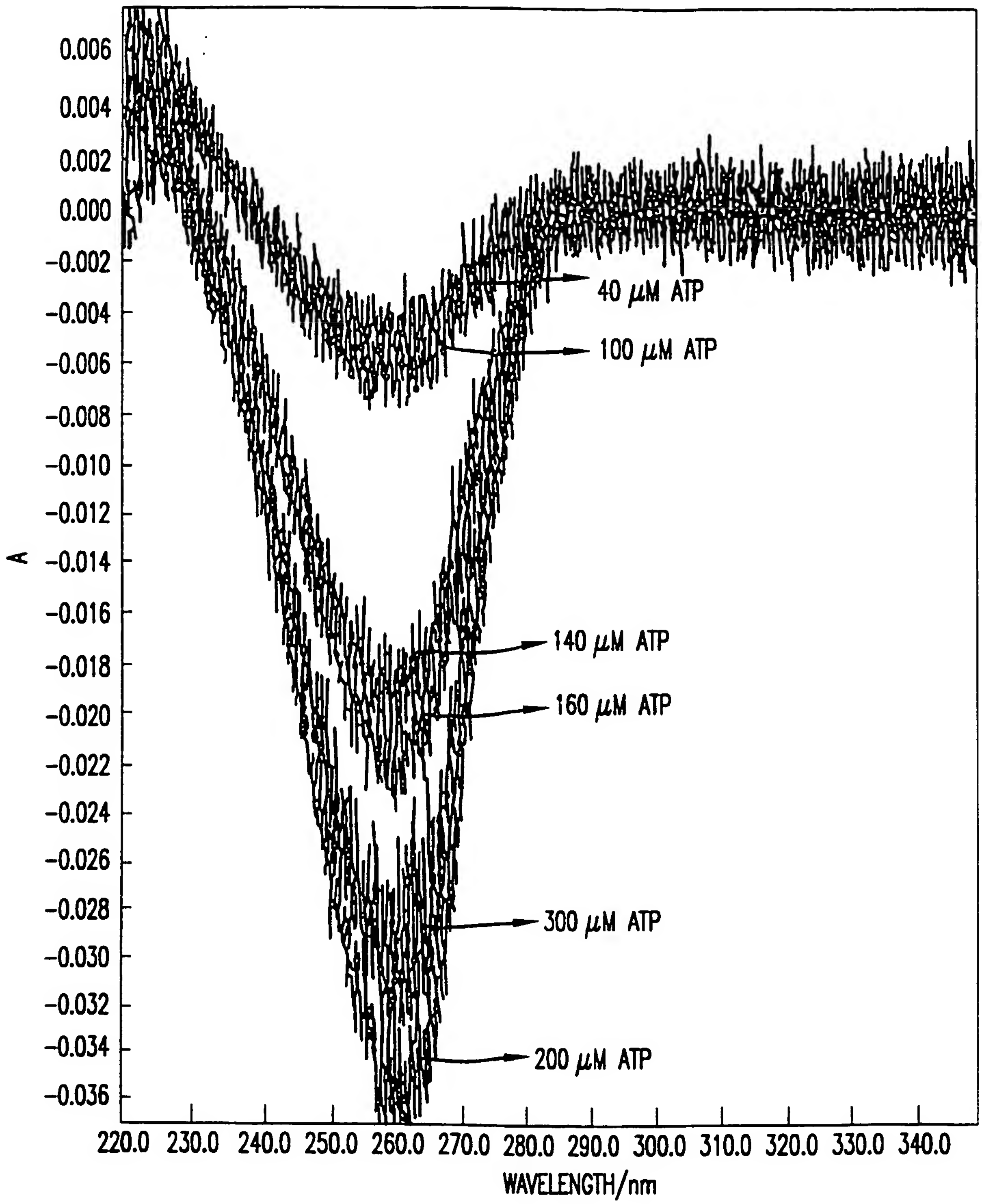

0.006
0.004
0.002
0.000
-0.002
-0.004
-0.006
-0.008
-0.010
-0.012
-0.014
-0.016
-0.018
-0.020
-0.022
-0.024
-0.026
-0.028
-0.030
-0.032
-0.034
-0.036
A
40 µM ATP
100 µM ATP
140 µM ATP
160 µM ATP
300 µM ATP
200 µM ATP
220.0
230.0
240.0
250.0
260.0
270.0
280.0
290.0
300.0
310.0
320.0
330.0
340.0
WAVELENGTH/nm

FIG.25

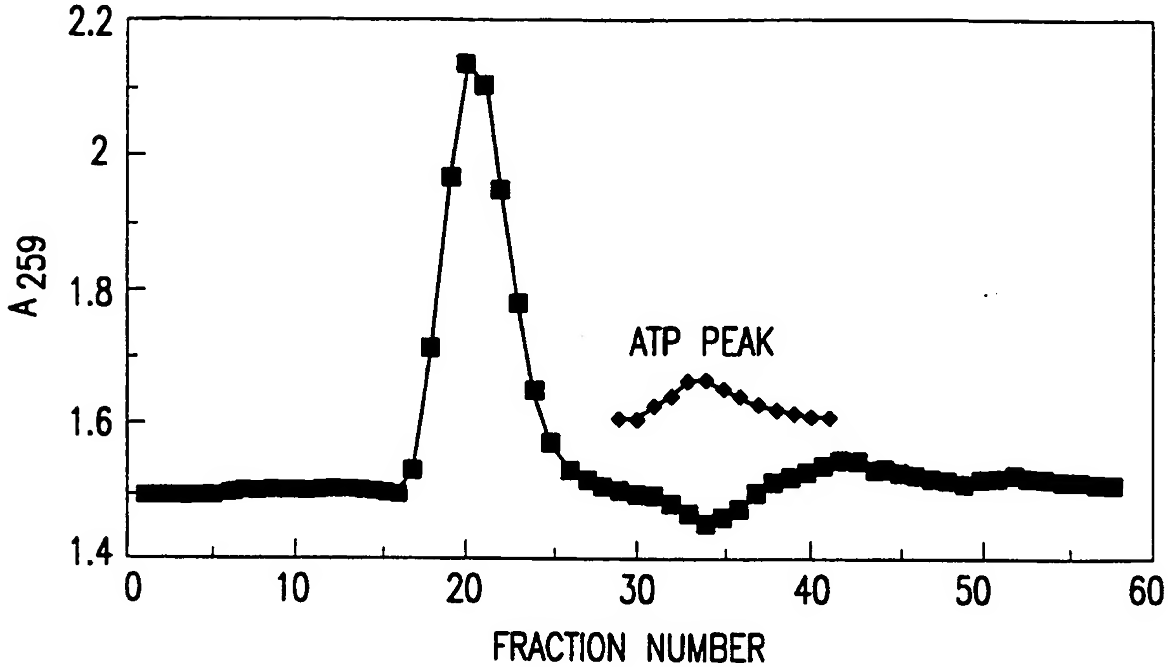

2.2
2
1.8
1.6
1.4
A_{259}
ATP PEAK
0
10
20
30
40
50
60
FRACTION NUMBER

FIG.26

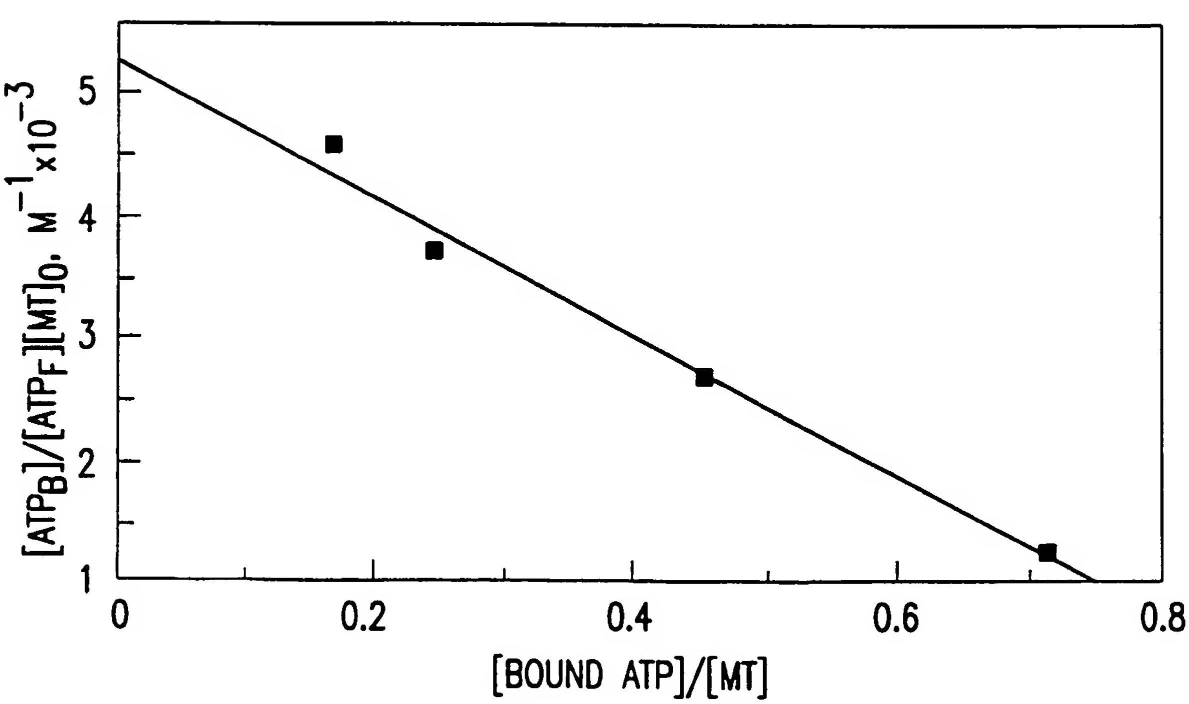

5
4
3
2
1
$[ATP_B]/[ATP_F][MT]_0,\ M^{-1}x10^{-3}$
0
0.2
0.4
0.6
0.8
[BOUND ATP]/[MT]

FIG.27

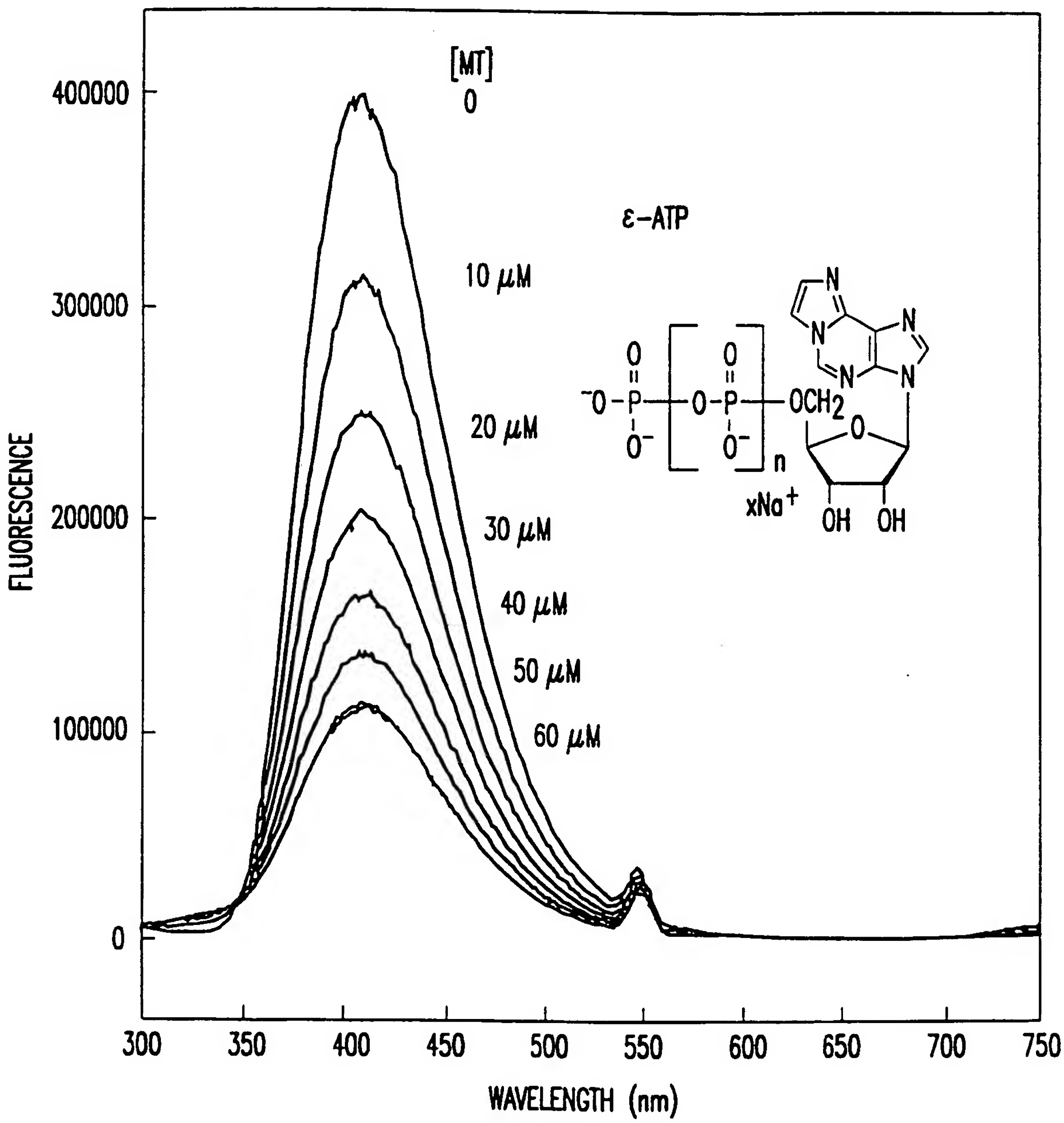

[MT]
0
10 μM
20 μM
30 μM
40 μM
50 μM
60 μM
ε-ATP
xNa+
n
OH OH
FLUORESCENCE
400000
300000
200000
100000
0
300
350
400
450
500
550
600
650
700
750
WAVELENGTH (nm)

FIG.28

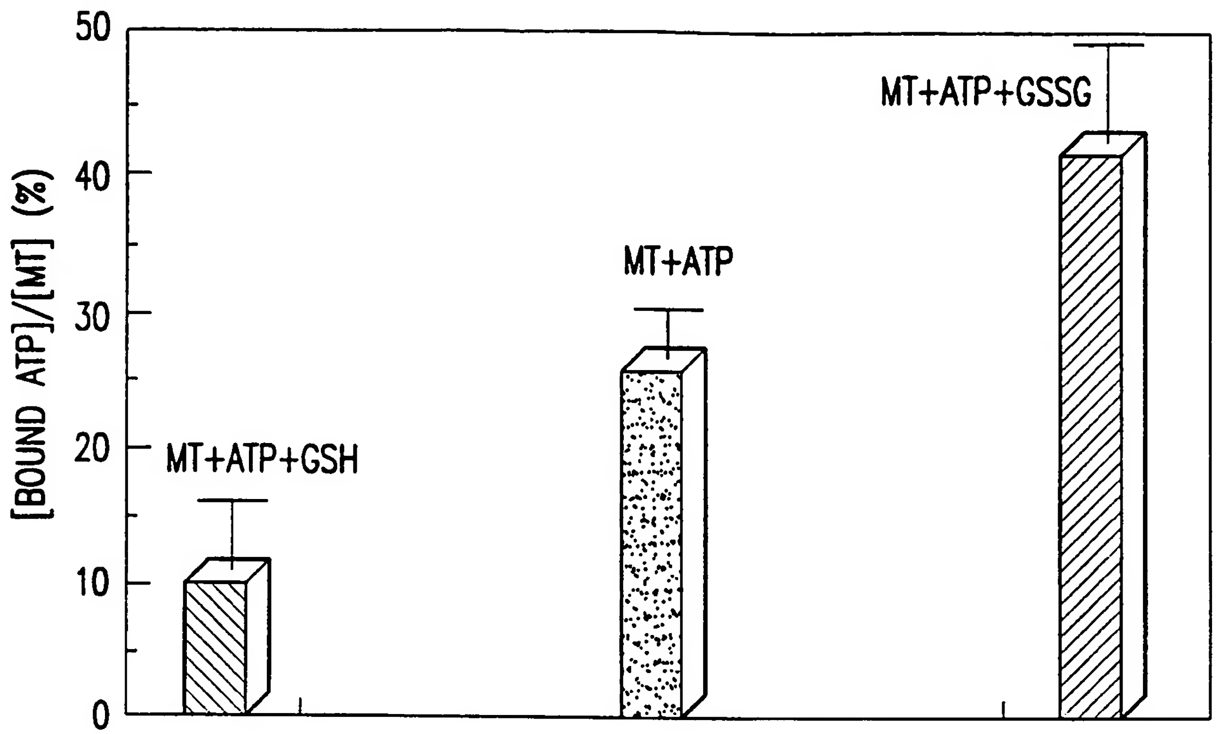


FIG.29

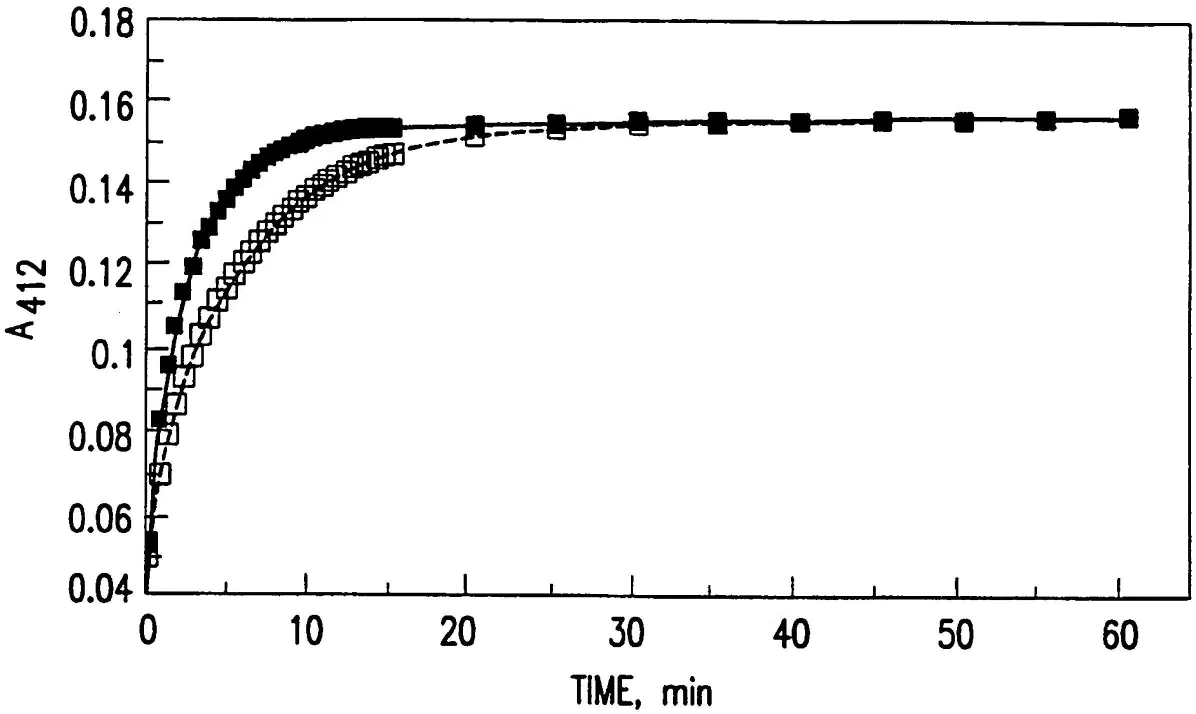


FIG.30A

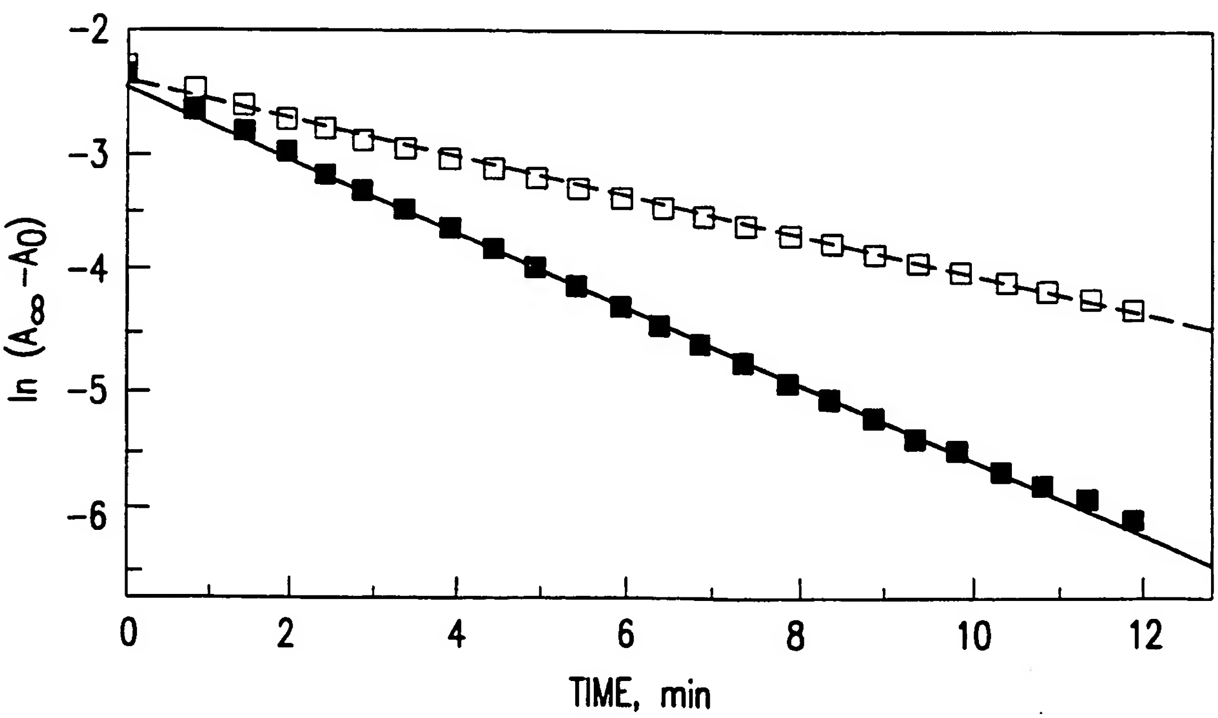


FIG.30B

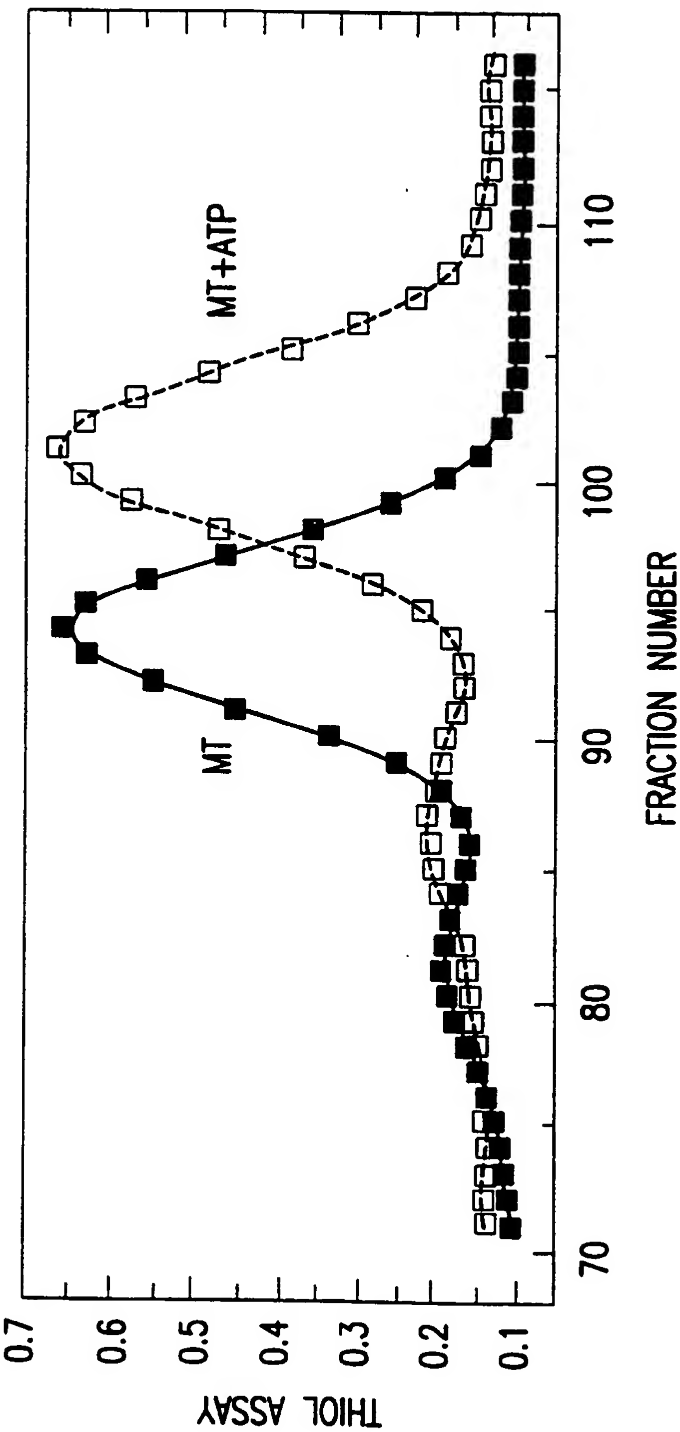

THIOL ASSAY
0.7
0.6
0.5
0.4
0.3
0.2
0.1
FRACTION NUMBER
70
80
90
100
110
MT
MT+ATP

FIG.31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07432

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/33, 31/355, 31/195
US CL :514/183, 458, 562
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/183, 458, 562

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAS-ONLINE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Medline on Dialog, Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, (Boston, MA 02115, USA), No. 95384026, MARET, W. 'Metallothionein/disulfide interactions, oxidative stress, and the mobilization of cellular zinc,' abstract, NEUROCHEMISTRY INTERNATIONAL,(July 1995) 27(1), pages 111-117	1-76
X	Database Medline on STN, Department of Pharmacology, University of Nebraska College of Medicine, (Omaha, Nebraska, 68198-6260, USA), No. 95384024, EBADI, M. et al. 'Expression and regulation of brain metallothionein,' abstract, NEUROCHEMISTRY INTERNATIONAL, (July 1995), 27(1), pages 1-22	1-76

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 JULY 1999	22 JUL 1999

Name and mailing address of the ISA/US	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07432

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US 5,849,290 A (BROWN et al.) 15 December 1998, see the entire document.	1-76